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$GABA_A$ receptors are differentially sensitive to zinc: dependence on subunit composition

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$GABA_A$ receptors with different subunit composition, were expressed in kidney cells and studied by whole cell recording. Expressed $GABA_A$ receptors were differentially sensitive to inhibition by zinc; receptors which lacked the γ subunit were inhibited by zinc. Embryonic neurones also exhibited zinc-sensitive GABA responses, in contrast to adult neurones. This developmentally-sensitive aspect of $GABA_A$ receptor pharmacology may be partly dependent on expression of the γ subunit.

Keywords: $GABA_A$ receptor; zinc; cloned GABA receptors; GABA receptor subunits; hippocampus; sympathetic ganglia; benzodiazepines; patch clamp

Introduction Zinc is a γ-aminobutyric acid (GABA) antagonist on cultured embryonic neuronal $GABA_A$ receptors (Westbrook & Mayer, 1987; Smart & Constanti, 1990). A comparison of embryonic and adult neurones, demonstrated that zinc inhibition of $GABA_A$ responses was correlated with the age of the neurones (reviewed in Smart, 1990). Embryonic neuronal GABA responses were consistently antagonized by zinc in a non-competitive manner, whereas adult neuronal responses were less sensitive and frequently insensitive to zinc antagonism (Smart & Constanti, 1990; Smart, 1990).

The application of cDNA cloning techniques to $GABA_A$ receptors has revealed a variety of different protein subunits (α, β, γ and δ) and also numerous subtypes of individual subunits (e.g. Verdoorn *et al.*, 1990). Functional $GABA_A$ receptors can be expressed in either *Xenopus* oocytes or mammalian cell lines using previously incorporated cRNAs or cDNAs. Homo-oligomeric GABA receptors can be formed from just one species of receptor subunit, or combinations of cRNAs and cDNAs can be used to construct hetero-oligomeric receptors (Malherbe *et al.*, 1990; Verdoorn *et al.*, 1990; Levitan *et al.*, 1988). The developmental aspect of $GABA_A$ receptor pharmacology, revealed by zinc, was conceivably due to different populations of $GABA_A$ receptors existing in embryonic and adult neurones, some sensitive and others insensitive to inhibition by zinc. This could reflect underlying structural differences between adult and embryonic $GABA_A$ receptors, perhaps due to differences in receptor subunit composition or differences in subunit processing.

This study investigated whether the antagonism of $GABA_A$ receptors by zinc was dependent on the receptor subunit composition.

Methods Human kidney cells (A293) were grown in Dulbecco's modified Eagles medium and Hams F12 with 10% foetal calf serum. Mouse $\alpha 1$, $\beta 1$ and $\gamma 2$ subunit cDNAs encoded polypeptides with amino acid sequences virtually identical to equivalent rat $GABA_A$ receptor polypeptides (100%, >99% and >99% respectively) (Kofuji *et al.*, 1991). The murine cDNAs were cloned as EcoR1 fragments into the mammalian expression vector p-Bex1 (British Biotechnology Ltd.). Expression was controlled by the human cytomegalovirus promoter. Kidney cells were transfected by a calcium phosphate technique (Moss *et al.*, 1990) and incubated at 37°C in 95% air:5% CO_2 for 48 h prior to electrophysiology. Cultured embryonic rat sympathetic ganglion (SCG) neurones were prepared as described previously (Smart & Constanti, 1990). Both SCG neurones and kidney cells were superfused at 30°C with Krebs solution containing (mM): NaCl 140, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11 and HEPES 5, pH 7.4. Whole-cell recordings were made with patch pipettes (1–5 MΩ) containing (mM): CsCl 120, tetraethylammonium-OH 33, $MgCl_2$ 1, $CaCl_2$ 1, EGTA 11 and HEPES 10, pH 7.1. Hippocampal brain slices were cut from young postnatal (P3–11 days; 600 μm thick) or adult ($P > 90$, 400 μm) rats and superfused at 30°C with Krebs containing (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2, $MgCl_2$ 2, $NaHCO_3$ 25, glucose 11, bubbled with 95% O_2:5% CO_2, pH 7.4. Intracellular recordings were made from CA3 pyramidal neurones with 3M KCl filled microelectrodes.

Results The expression of $GABA_A$ receptors in cell lines formed from different subunit combinations which included an α subunit, generally produced large GABA-induced membrane currents (Moss *et al.*, 1990; Verdoorn *et al.*, 1990). In contrast, receptors lacking an α subunit or homo-oligomeric GABA receptors, produced much smaller GABA-evoked currents making analysis difficult (Levitan *et al.*, 1988; Verdoorn *et al.*, 1990). Therefore, we used two types of $GABA_A$ receptor, formed from either $\alpha 1\beta 1$, or $\alpha 1\beta 1\gamma 2$ subunit combinations. Zinc (50–300 μM) produced virtually no inhibition of the GABA-induced current when whole cell recording from clusters of cells, or single cells, transfected with $\alpha 1\beta 1\gamma 2$ cDNAs (Figure 1a). In contrast, GABA responses mediated by receptors comprising just $\alpha 1\beta 1$ subunits, were clearly inhibited by zinc in a reversible manner (Figure 1b). GABA concentration-response curve analysis revealed a non-competitive inhibition by zinc on $\alpha 1\beta 1$ $GABA_A$ receptors (Figure 1c) with little variation with the agonist concentration (mean inhibition $82 \pm 6\%$; 0.5–10 μM GABA). The IC_{50} for zinc was estimated as 1.5 μM from the zinc inhibition curve (Figure 1d).

The insensitivity to zinc of $GABA_A$ receptors containing the $\gamma 2$ subunit was interesting, since the inclusion of this subunit in the receptor structure also confers a sensitivity to benzodiazepines (BDZ) (Pritchett *et al.*, 1989; Figure 1a). $\alpha 1\beta 1$ expressed receptors are either insensitive to BDZ (Figure 1b), or display an unusual BDZ pharmacology (Pritchett *et al.*, 1989; Malherbe *et al.*, 1990; Moss *et al.*, 1990). We predicted that $GABA_A$ receptors which are insensitive to zinc, should also be sensitive to BDZ and *vice versa*. Accordingly, we investigated the effect of zinc on cultured SCG neurones and pyramidal neurones in hippocampal brain slices. In the same SCG neurones, GABA responses were antagonized by 100 μM zinc and also enhanced by 1–10 μM flurazepam. Zinc still produced a similar percentage inhibition of the GABA response, even in the presence of flurazepam (Figure 2a).

In contrast, GABA responses recorded from young postnatal (P11) and adult (P > 90) hippocampal brain slices were not

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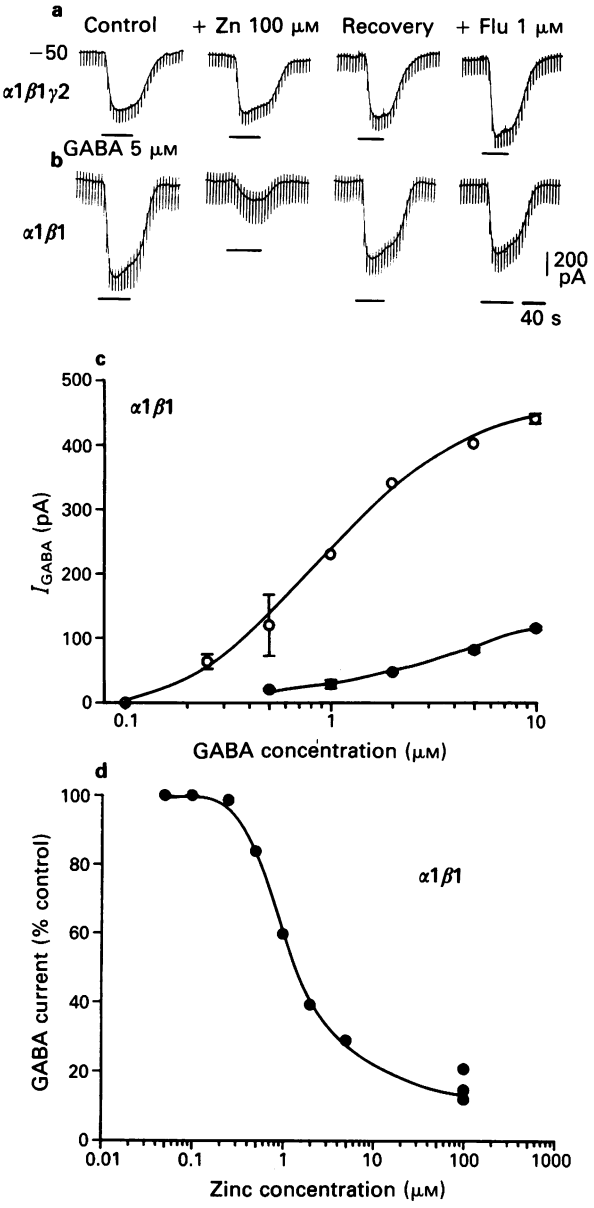

Figure 1 Zinc antagonism of GABA-activated membrane currents mediated by cloned $GABA_A$ receptors of different combinations. (a,b) Whole-cell currents (I_{GABA}) were recorded at −50 mV from cells transfected with $\alpha1\beta1\gamma2$, or $\alpha1\beta1$ GABA cDNAs in the presence and absence of 100 μM zinc, or 1 μM flurazepam (Flu). Brief hyperpolarizing voltage commands were used to monitor membrane conductance (200 ms, −10 mV, 0.2 Hz). GABA 5 μM was bath-applied for the duration indicated by the solid line. (c) Effect of zinc on the GABA concentration-response relationship in $\alpha1\beta1$ transfected cells. GABA responses are plotted as means of 3–5 determinations of the peak I_{GABA} in control (○) and in the presence of 100 μM zinc (●); vertical bars show s.e.mean. (d) Inhibition plot for zinc antagonism of GABA responses mediated by $\alpha1\beta1$ receptors. Peak I_{GABA} was measured to 5 μM GABA in the absence and presence of different concentrations of zinc.

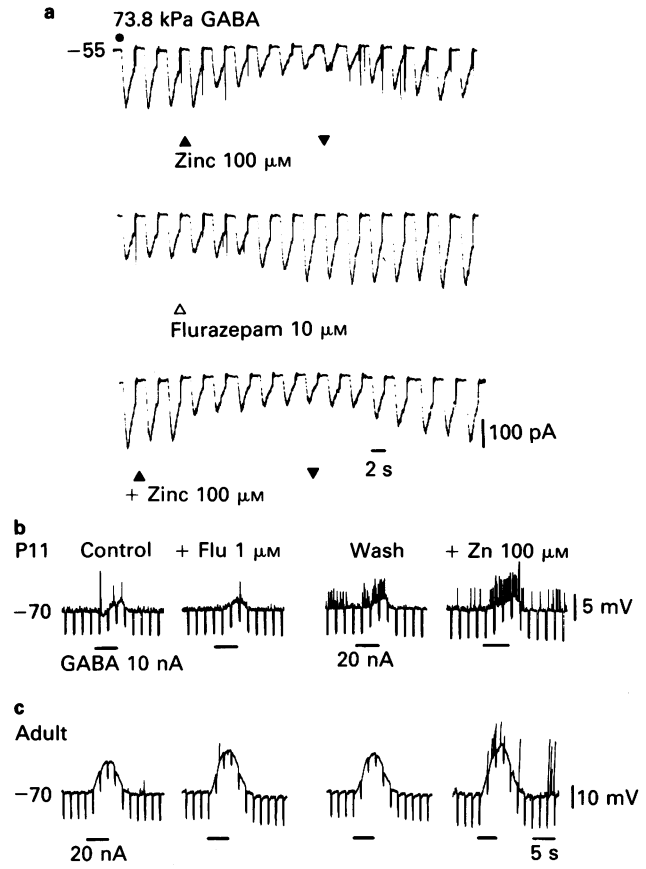

Figure 2 Effect of zinc and flurazepam on GABA-activated membrane currents in vertebrate neurones. (a) Whole-cell I_{GABA} currents were recorded from a cultured superior cervical ganglion neurone (E21) at −55 mV. All three traces represent a continuous recording. GABA responses evoked by pressure application (73.8 kPa; 30 ms; 1 pulse $30\,s^{-1}$) were inhibited by zinc 100 μM. Flurazepam (Flu 10 μM) enhanced GABA responses but subsequent addition of zinc in Flu still produced a comparable inhibition in I_{GABA}. (b,c) GABA-activated depolarizations produced by ionophoretically-applied GABA to hippocampal CA3 neurones. Small hyperpolarizing current pulses (−0.4 nA; 300 ms; 0.5 Hz) monitored membrane resistance. Flu and zinc were bath-applied to young postnatal (P11) (b) and adult (c) neurones. Note Flu produced a clearly enhanced GABA response only in the adult neurones and zinc was ineffective as a GABA antagonist in slices of either age.

blocked by zinc but slightly enhanced, mainly due to a reduction in the resting membrane input conductance (Smart & Constanti, 1990) (Figure 2b). However, in young postnatal slices, flurazepam (0.5–10 μM; $n = 6$) rarely produced any enhancement of the GABA response, but in adult slices, the sensitivity of the GABA response to BDZ became readily apparent (Figure 2c).

Discussion The inhibition of GABA responses by zinc clearly depends on the composition of the $GABA_A$ receptor as receptors possessing a $\gamma2$ subunit are relatively insensitive to blockade by zinc ions. In contrast, expressed $GABA_A$ receptors composed of $\alpha1\beta1$ subunits, were sensitive to zinc inhibition. Since GABA responses from embryonic neurones are routinely zinc-sensitive (Smart & Constanti, 1990; Smart, 1990), the major type of $GABA_A$ receptor present at this stage of neural development might be lacking a $\gamma2$ subunit. Adult and older postnatal forms of the $GABA_A$ receptor would then presumably contain the $\gamma2$, or a functionally equivalent subunit, thereby rendering the receptor insensitive to zinc. However, GABA responses recorded from SCG neurones, retained a sensitivity to both BDZ and zinc in the same cell suggesting that $GABA_A$ receptors with and without the $\gamma2$ subunit were present. Vertebrate neurones are likely to possess a heterogeneous population of $GABA_A$ receptors, and possibly both zinc-sensitive and -insensitive $GABA_A$ receptors may co-exist in the same cell with the balance between these receptor populations varying between embryonic and adult neural tissues.

GABA responses recorded from adult CA3 pyramidal neurones in brain slices were readily enhanced by BDZ and not blocked by zinc, as expected if the major type of $GABA_A$ receptor in adult neural tissues contains the $\gamma2$ subunit; however, our results with young CA3 neurones ($P < 11$) indicated that these GABA responses are apparently insensitive to BDZ (Rovira & Ben-Ari, 1990), and also surprisingly insensitive to zinc blockade. If the $GABA_A$ receptor in young CA3

neurones lacks the $\gamma 2$ subunit, then some susceptibility to zinc antagonism would have been expected.

Zinc sensitivity of the $GABA_A$ receptor is apparently determined by the subunit composition and this, in part, is influenced by the absence of the $\gamma 2$ subunit. However, the results with young hippocampal brain slices suggested other combinations of $GABA_A$ receptor subunits which apparently lack the $\gamma 2$ subunit are also likely to be insensitive to zinc.

The differential pharmacology of the $GABA_A$ receptor protein complex towards zinc, which is apparently related to neuronal development, is a novel finding for this receptor and may be useful in probing the subunit composition of different $GABA_A$ receptor subtypes in many other areas of the CNS.

Note added in proof

Following the original submission of this paper, a study by Draguhn and colleagues (1990, *Neuron.*, **5**, 781–788) using recombinant rat $GABA_A$ receptors also revealed that receptors containing the $\gamma 2$ subunit were relatively less sensitive to inhibition by zinc.

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Human, pig and guinea-pig bladder smooth muscle cells generate similar inward currents in response to purinoceptor activation

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The contribution of purinergic neurotransmission to bladder excitation in pigs and man is small. Exogenously-applied adenosine-trisphosphate (ATP) however, elicits large inward currents in dispersed bladder smooth muscle cells in both species. The essential properties of the ATP-induced current in human and pig detrusor are similar and the current intensity is comparable to those in the guinea-pig, which has a powerful purinergic excitatory innervation. This suggests that other features of the tissue such as the closeness of the innervation and the degree of cell-to-cell coupling may be important in determining the effectiveness of purinergic transmission.

Keywords: Smooth muscle; ATP; urinary bladder; purinoceptors; purinergic transmission

Introduction The urinary bladder of small mammals receives a dual excitatory innervation from cholinergic and non-adrenergic, non-cholinergic (NANC) nerves. Purinergic transmission is probably responsible for the latter, as demonstrated by various functional (Burnstock *et al.*, 1978) and electrophysiological studies (Fujii, 1988). The relative contribution of cholinergic and purinergic transmission varies markedly amongst species, the purinergic contribution being 70–80% at high frequencies in guinea-pigs (Inoue & Brading, 1990) and 15% in pigs (Sibley, 1984). An atropine-resistant tetrodotoxin-sensitive contribution has not been found in normal human bladders from cadaver donors (Sibley, 1984), although a small NANC component has been seen occasionally in some strips from patients with bladder disorders (Sjögren *et al.*, 1982; Hoyle *et al.*, 1989; Luheshi & Zar, 1990; Ruggieri *et al.*, 1990). The differences in the purinergic innervation could be caused by differences in the postsynaptic purinoceptors, or by differences in the organization and innervation of the bladder. In this short paper, in order to distinguish between these possibilities, we describe the effects of postsynaptic purinoceptor activation on membrane currents in single cells from the detrusor of guinea-pigs, pigs and man, and conclude that any small inter-species differences present cannot account for the different degrees of purinergic transmission in the whole animal.

Methods Urinary bladders of Landrace pigs were obtained from a slaughter house and human bladders from patients undergoing cystectomy for bladder cancer at the John Radcliffe Hospital, Oxford. In the latter case, only tumour-free areas of the bladder wall were used, and the results in Table 1 are from four bladders. After preincubation in Ca-free solution, small pieces of detrusor were digested in Ca-free solution supplemented with 0.2% collagenase (Sigma type I and Boehringer) and 0.2% pronase (Serva, type E) for 30–45 min at 36°C with continuous stirring. The resultant cell suspension was diluted 1:1 in modifed Krebs solution as previously described (Inoue & Brading, 1990).

The physiological saline solution contained (mM): Na^+ 140, K^+ 6, Ca^{2+} 2, Mg^{2+} 1.2, Cl^- 152.4, glucose 10 and HEPES 10, titrated to pH 7.35–7.40 with Tris base. Ca-free solution was prepared by omitting Ca and Mg from the solution. The internal pipette solution contained (mM): Ca^+ 140, Mg^{2+} 2, Cl^- 144, HEPES 10 and EGTA 1 or 10, titrated to pH 7.2 with Tris base. The recording system was the same as previously described (Inoue & Brading, 1990). For analysis, data were digitized through an A/D converter (DT2801A, Data Translation) under the control of an IBM compatible computer using a programme written in Quick Basic 6.0. For illustrations, an intelligent digital plotter (HP7475A, Hewlett Packard) was used. ATP was made up just before use and applied by use of a concentration-jump apparatus (Inoue & Brading, 1990).

Results Single cells obtained from human or porcine detrusor appear bigger than those from the guinea-pig and have a larger surface area as estimated from the input capacitance (Table 1). The majority of cells contracted immediately on exposure to ATP. When cells were clamped at −60 mV, rapid application of ATP elicited a fast activating, rapidly desensitizing inward current with a very short latency (less than 100 ms) in a concentration-dependent manner in all cells studied (Figure 1). The properties of the ATP-induced inward current are summarized for each species in Table 1. The maximum intensity of the ATP-induced inward current did not differ significantly between the three species. However, half-maximal activation of the current required somewhat higher concentrations for pig and human detrusor than for

Table 1 Characteristics of ATP-induced inward currents recorded in porcine, human and guinea-pig detrusors

	Pig	*Man*	*Guinea-pig*
Input capacitance (pF)	80 ± 10 (n = 10)	106 ± 27 (n = 8)	49 ± 14*
Maximum current intensity (μA/μF)	6.7 ± 4.2 (n = 6)	21.1 ± 18.7 (n = 6)	19.6 ± 23.4
50% activation (μM)	3–10 (n = 3)	10–30 (n = 5)	2.3*
Reversal potential (mV)	+1.4 ± 2.3 (n = 3)	+3.2 ± 1.8 (n = 4)	+1.2 ± 1.9*

The maximum current intensity was calculated by dividing the maximum current amplitude recorded at −60 mV by the input capacitance; 50% activation and reversal potentials were estimated from a series of recordings from the same cell by changing the concentration of ATP or the holding potential, respectively.
The data with a * are taken from Inoue & Brading (1990) (n = 21).

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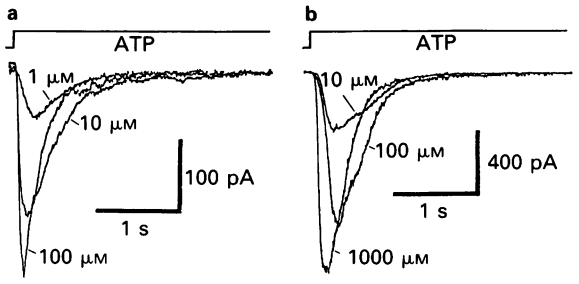

Figure 1 ATP activates large inward currents in pig (a) and human (b) detrusor. The currents recorded at three different concentrations of ATP are superimposed and labelled. ATP was applied by a concentration-jump method and was present throughout each record. Note the rapid activation and inactivation of the inward current, and the difference in the current scale on the two panels.

guinea-pig, although the limited number of trials with the large mammals precludes exact evaluation. The reversal potentials were not significantly different, suggesting similar ionic selectivity of the channels in the three species. In addition, the current was greatly reduced by removal of extracellular Na and was virtually abolished in the absence of Ca and Na (not shown). Insensitivity to a high concentration of intracellular Ca buffer (10 mM EGTA) was also observed among all species and α,β-methylene ATP blocked the response to a following application of ATP in both guinea-pig and pig.

Discussion The present results strongly suggest that the smooth muscle cells of the detrusor of the three species studied all possess a high density of ATP receptors, activation of which can generate large inward currents. The currents have remarkably similar properties, and the small differences recorded in their sensitivity to ATP cannot account for the weakness of the purinergic innervation in the pig, and it certainly cannot account for the frequent lack of demonstrable purinergic innervation in human bladder strips.

One explanation could be that ATP is not released from intrinsic nerves in man, whilst varying amounts are released in other species. However, ATP is normally thought to be co-released with acetylcholine from peripheral cholinergic nerves, and all three species have a relatively dense and uniform distribution of cholinesterase-positive intrinsic fibres. There is little firm evidence to suggest the existence of separate purinergic nerves. It is thus perplexing that with a large population of ATP-activated channels and a parasympathetic innervation, a purinergic component is either small or absent in human bladder in response to nerve stimulation.

The explanation may lie in differences in the detailed anatomy of the innervation, combined with the rapid desensitization of the ATP-activated channels and poor electrical coupling between the smooth muscle cells in the detrusor. A relatively high concentration of ATP is needed to activate the channels, and if the varicosities releasing transmitter vary in their closeness to the smooth muscle, ATP will undergo diffusional dilution and hydrolysis before reaching the receptors. A slow rise in the concentration of ATP at the receptors may also desensitize them before significant activation occurs. If only a small proportion of cells is innervated, poor electrical coupling would also restrict the spread of excitation. An increase in the cell-to-cell coupling as has been suggested in detrusor instability (Foster *et al.*, 1989) might account for the appearance of a purinergic component of excitatory innervation in this condition (Sjögren *et al.*, 1982).

In conclusion, the variations in the purinergic component of excitatory innervation seen in bladder smooth muscles of different species, cannot be accounted for by differences in the properties or density of the postsynaptic purinoceptors.

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Nedocromil sodium inhibits airway hyperresponsiveness and eosinophilic infiltration induced by repeated antigen challenge in guinea-pigs

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1 Repeated exposure to ovalbumin aerosol produced significant increases in epithelial eosinophils of airways of all sizes and produced increased pulmonary resistance (R_L) to inhaled acetylcholine (ACh) in guinea-pigs.

2 Nedocromil sodium 10 mg ml^{-1}, by nebulization prior to each ovalbumin (OA) challenge inhibited both the airway eosinophilia and hyperresponsiveness to ACh.

3 Nedocromil sodium pretreatment (10 mg ml^{-1} by nebulization) 10 min prior to OA completely inhibited the acute bronchoconstrictor response to OA.

4 Our findings suggest that nedocromil sodium inhibits airway hyperresponsiveness by inhibiting eosinophilic infiltration, or by simultaneously inhibiting mechanisms involved in both processes.

Keywords: Airway hyperresponsiveness; asthma; eosinophilia; nedocromil sodium

Introduction

The development of non-specific airway hyperresponsiveness is a hallmark of bronchial asthma (Cockroft *et al.*, 1977). We have developed a model of guinea-pigs which demonstrates a number of features of bronchial asthma including acute bronchoconstrictor responses to antigen, airway eosinophilia and airway hyperresponsiveness (Ishida *et al.*, 1989).

The general belief that eosinophils are causally related to airway hyperresponsiveness was questioned by our recent finding that pretreatment with a platelet-activating factor antagonist prior to each of the multiple antigen challenges produced striking inhibition of the airway hyperresponsiveness to inhaled acetylcholine but failed to alter the acute bronchoconstrictor responses or the airway eosinophilia (Ishida *et al.*, 1990). Since eosinophils are a source of platelet-activating factor (Lee *et al.*, 1990), these data may suggest that the antagonist worked at a post-eosinophil step in the development of airway hyperresponsiveness. Alternatively, airway eosinophils may not be directly linked to airway hyperresponsiveness.

Since nedocromil sodium has been shown to inhibit antigen-induced early and late bronchoconstrictor responses plus bronchoalveolar lavage eosinophilia in guinea-pigs (Hutson *et al.*, 1988) and man (Crimi *et al.*, 1989) in addition to decreasing airway hyperresponsiveness in grass pollen-sensitive individuals during the pollen season (Dorward *et al.*, 1986), we chose to evaluate the effects of this drug upon both airway hyperresponsiveness and airway eosinophilia induced by repeated antigen challenge.

Methods

Cam-Hartley female guinea-pigs were sensitized to ovalbumin by inhalation of an aerosolized solution of 1% ovalbumin (Sigma Chemical Company, St. Louis, MO, U.S.A.) dissolved in sterile, non-pyrogenic normal saline containing 4% heat-killed *Bordetella pertussis* vaccine (Connaught Labs, Toronto, Ontario) according to a previously described protocol (Ishida *et al.*, 1989). Sensitizations and challenges were performed with a DeVilbiss ultrasonic nebulizer (mass median particle diameter 9.6 μm, output 7.2 ml min^{-1}).

Sensitized guinea-pigs were subjected to either acute or chronic antigen challenges with aerosolized ovalbumin 0.5%. Control animals received aerosolized normal saline in an identical manner. Nedocromil sodium, 10 mg ml^{-1}, by nebulization for 3 min was given 5 min prior to antigen exposure. All groups of animals received diphenyhydramine (Sigma Chemical Company, St. Louis, MO) 20 mg kg^{-1} i.p., one hour before antigen or saline exposure.

To determine the effects of nedocromil sodium on the acute antigen-induced bronchoconstriction, eight guinea-pigs weighing approximately 500 g were sensitized, sensitization confirmed by a single challenge with 0.5% ovalbumin aerosol one week later and then evaluated for ovalbumin-induced changes in pulmonary resistance (R_L) three days later. Four of the animals received nebulized nedocromil sodium (10 mg ml^{-1}) for 3 min before being anaesthetized with pentobarbitone sodium (Abbott Labs, Montreal, Canada), and tracheostomized; a fluid-filled oesophageal line was inserted and the animal was placed in a body plethysmograph containing a copper wire mesh heat sink. Animals were paralyzed with an intramuscular injection of succinylcholine, 0.5 mg kg^{-1}, and ventilated with a Harvard animal ventilator (Model 608, Harvard Apparatus Company, South Natwick, MA, U.S.A.) delivering a tidal volume of 3 ml at a frequency of 60 breaths min^{-1}. Transpulmonary pressure was determined by comparing pressure at the tracheal opening with the oesophageal pressure measured with a saline-filled PE-90 tube in the distal oesophagus. Flow, volume and pressure signals were recorded and R_L was calculated from these signals by the method of von Neergard & Wirz (1927) and a computer programme developed in this laboratory (Hulbert *et al.*, 1985). After baseline values were stabilized animals were given six tidal volume breaths of 0.5% ovalbumin by an acorn nebulizer in a flow of 5 litres room air. R_L determinations were made 15 s, 3 min and 10 min after the initial ovalbumin exposure, followed by six tidal volume breaths of nebulized 5.0% ovalbumin with repeat R_L measurements at 15 s, 3 min and 10 min.

For chronic studies of repeated antigen challenge, 30 guinea-pigs weighing 250 to 300 g were sensitized to ovalbumin and divided into three groups. Control animals received subsequent challenges with aerosolized saline rather than ovalbumin. All other animals received 0.5% ovalbumin in 10 min exposures twice weekly for 4 to 5 weeks. Ten of these were pretreated with nedocromil sodium (10 mg ml^{-1}) by nebulization for 3 min in an exposure chamber, 10 min before exposure to ovalbumin challenge.

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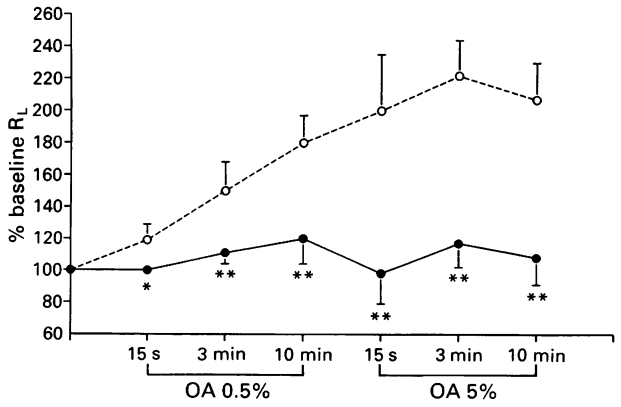

Figure 1 Changes in pulmonary resistance (R_L) following 6 tidal breaths of nebulized ovalbumin (OA) in guinea-pigs previously sensitized to ovalbumin. Values are means with s.e. shown by vertical bars for untreated control guinea-pigs (○, $n = 4$) and for those receiving nedocromil sodium 10 mg ml^{-1} for 3 min by nebulizer 10 min before OA challenge (●, $n = 4$). * and ** indicate $P < 0.05$ and $P < 0.01$ in comparison with control group. The baseline R_L values (100%) for groups not receiving and those receiving nedocromil sodium were 0.141 ± 0.008 and 0.140 ± 0.017 cmH_2O ml^{-1} s^{-1}.

The acetylcholine (ACh) dose-response curve was measured 3 days after the last ovalbumin challenge. Guinea-pigs were anaesthetized, paralyzed and ventilated in the body plethysmograph as for the acute challenge. Following baseline R_L determinations, six tidal volume breaths of normal saline were

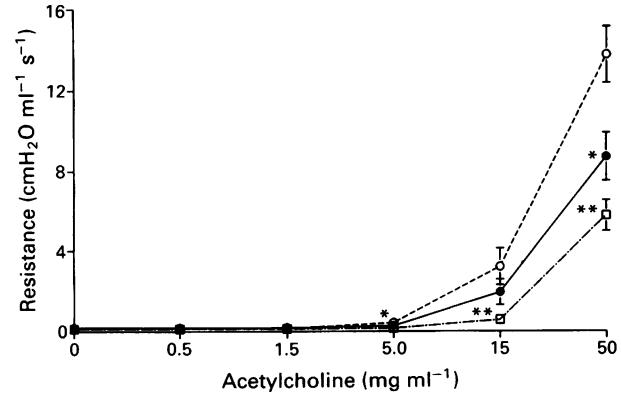

Figure 2 Changes in pulmonary resistance with increasing doses of aerosolized acetylcholine (ACh) in saline-challenged control guinea-pigs (□, $n = 21$), and in guinea-pigs receiving repeated aerosol ovalbumin challenges with (●, $n = 10$) nebulized nedocromil sodium 10 mg ml^{-1} pretreatment with or without (○, $n = 36$) pretreatment. * and ** indicate $P < 0.05$ and $P < 0.01$ in comparison with animals receiving ovalbumin without drug pretreatment. Baseline values are shown in Table 1. Both the saline (□) and nedocromil sodium pretreated (●) groups were significantly different from the ovalbumin group at 5 mg ml^{-1} ACh.

given followed by increasing doses of ACh given in the same manner. Between doses, time was allowed for baseline resistance to return to normal, before the next ACh dose. The ACh doses provoking 5, 10, 20 and 30 fold increases from baseline R_L were calculated for each guinea-pig (PC_5, PC_{10}, PC_{20}, PC_{30} respectively) by extrapolation from the individual dose-response curves, and the geometric mean value for each group compared. Statistical analysis were performed by multivariate repeated measures analysis of variance (Fleiss, 1985).

For histological evaluation, the lungs and tracheas from freshly killed guinea-pigs were inflated with fixative (2.5% glutaraldehyde in 0.1% M cacodylate buffer) to 15 cmH_2O pressure and then immersed in the fixative. Tissue samples were taken from the trachea, left and right main bronchi, left upper and lower lumbar bronchi, right middle and lower bronchi and the parenchyma of the same lobes. Tissue samples were dehydrated in a graded series of ethanols, embedded in glycol methacrylate (JB-4, PolySciences, Warrington, PA, U.S.A.) and 3 μm sections were cut, mounted on glass slides and stained with either toluidine blue O or a Hansel's staining technique with slight modifications (Nolan *et al.*, 1986). By use of a digitizer (Digi-Pad Type 5, GTCO Corp., Brockville, MD, U.S.A.) and a programme developed in this laboratory (James *et al.*, 1988), the internal perimeter of the airway (marked by the epithelial luminal surface) and the basal surface of epithelial cells were measured for determination of epithelial tissue area using slides stained with toluidine blue O. Airway size was determined by the internal perimeter as outlined by James *et al.* (1988). Eosinophils were counted in the epithelial and connective tissue compartments using the Hansel's stained slides. Morphometric parameters of the three groups were compared to various airway sizes by random effects linear regression analysis (Feldman, 1988).

Results

Acute allergen challenge

Guinea-pigs, sensitized to ovalbumin, developed visible respiratory difficulty as shown by increased respiratory rate, indrawing and cyanosis with antigen challenge. The acute responses to inhaled antigen given as six tidal breaths to anaesthetized, paralyzed and tracheostomized animals are shown in Figure 1. Both groups of animals received identical ovalbumin challenges with one group receiving nedocromil sodium pretreatment. Pretreatment with nedocromil sodium prevented the increase in pulmonary resistance which occurred following the two doses of antigen inhaled and the difference between the groups was highly significant at all time points evaluated.

Airway hyperresponsiveness

Figure 2 compares the responses to ACh of guinea-pigs repeatedly challenged with ovalbumin both with and without

Table 1 Baseline pulmonary resistance (R_L) and provocative concentrations (PC) of acetylcholine

	Baseline R_L (cmH_2O ml^{-1} s^{-1})	*PC_5*	*PC_{10}*	*PC_{20}*	*PC_{30}*
Control ($n = 21$)	0.124 ± 0.006	13.9** (12.1,15.9)	18.6** (16.7,20.8)	26.7** (23.6,30.2)	31.9** (28.0,36.3)
Ovalbumin + nedocromil sodium ($n = 10$)	0.184 ± 0.022	11.7** (8.5,16.0)	15.1** (11.0,20.5)	22.3** (16.4,30.3)	25.3** (18.7,34.2)
Ovalbumin ($n = 36$)	0.143 ± 0.007	7.6 (6.0,11.6)	9.6 (6.3,14.6)	12.7 (8.3,19.4)	16.5 (11.5,23.6)

Baseline R_L values are arithemtic means ± s.e. PC values are geometric means (95% confidence limits) producing 5 fold (PC_5), 10 fold (PC_{10}), 20 fold (PC_{20}) and 30 fold (PC_{30}) increases from baseline R_L.
** indicates $P < 0.01$ as compared with ovalbumin group.

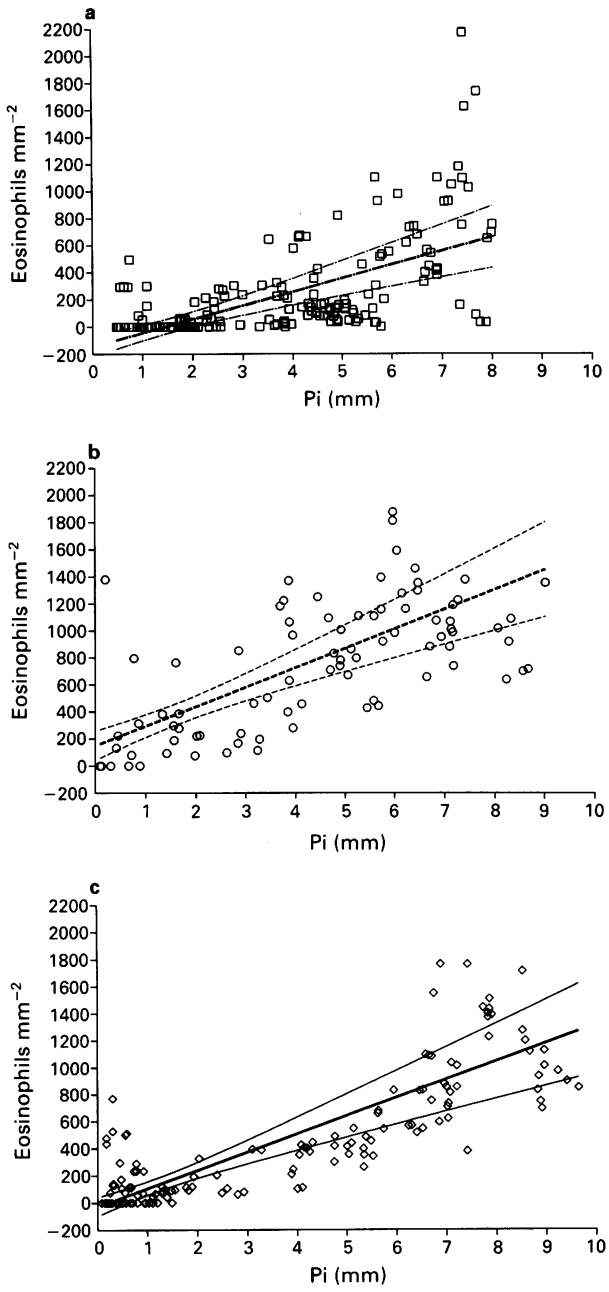

Figure 3 Airway epithelial eosinophils (number mm^{-2}) for varying airway size based upon the internal perimeter of epithelium (Pi) where Pi = 6.2–8.7 mm for tracheal, 5.8–7.9 mm for main bronchi, 3.7–6.9 and 0.7–3.4 mm for small airways. Data points plus the mean regression line and its 95% confidence intervals of individual regression lines for each guinea-pig are shown for: (a) control guinea-pigs receiving repeated saline challenges, $n = 11$; (b) guinea-pigs repeatedly challenged with ovalbumin, $n = 6$; (c) guinea-pigs repeatedly challenged with ovalbumin following pretreatment with nedocromil sodium, $n = 7$.

receiving nedocromil sodium prior to each ovalbumin challenge, as well as in a control group receiving normal saline rather than ovalbumin. The increased responsiveness to ACh of the ovalbumin challenged animals was comparable to that seen in previous studies (Ishida *et al.*, 1989; 1990). Statistical analysis of the present and historical data indicated no significant differences within either saline control groups or ovalbumin-challenged groups. Since the only differences are in the time at which experiments were performed, and because we have no reason to believe that there have been temporal changes in the laboratory, we have considered the present and historical data for the control and ovalbumin groups as single groups of 21 and 36 animals respectively. These were compared with 10 animals receiving nedocromil sodium pretreatment before each of the ovalbumin challenges. Responses of the nedocromil sodium-treated group lay between those for ovalbumin-challenged and saline control, being significantly different ($P < 0.05$) at 5 mg ml^{-1} and 50 mg ml^{-1} ACh when compared with the ovalbumin-challenged group. More significant differences ($P < 0.001$) occurred for the saline control group versus ovalbumin-challenged group at doses of 15 and 50 mg ml^{-1}, ACh.

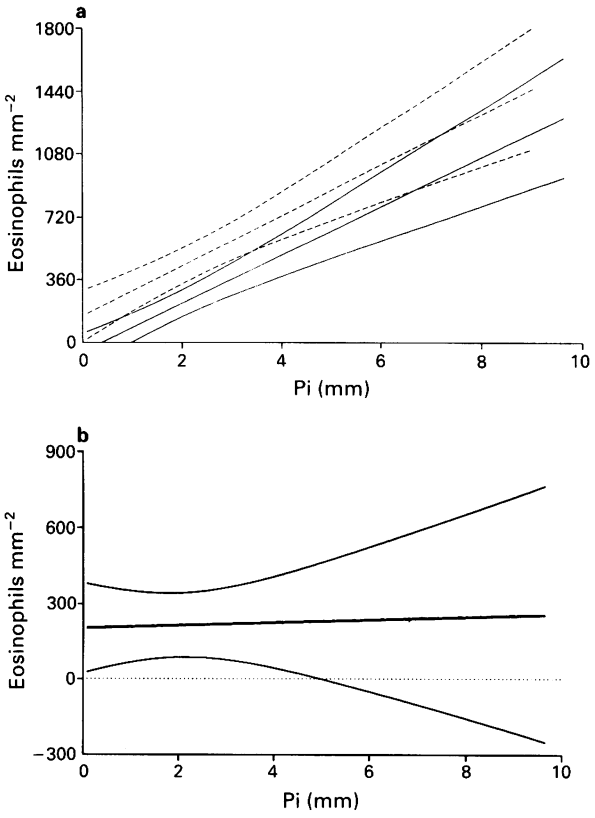

Figure 4 (a) Airway epithelial eosinophils for all airway sizes (as in Figure 3): 95% confidence limits of the regression analysis are depicted for airways of guinea-pigs receiving repeated ovalbumin challenges with (——) or without (– – –) nedocromil sodium pretreatment. (b) Differences in airway epithelial cells for the groups shown in (a). Ovalbumin-challenged animals demonstrate greater numbers of eosinophils in small airways and lobar bronchi compared with nedocromil sodium pretreated, ovalbumin-challenged animals. Pi values are in Figure 3.

When evaluated as the provocative concentration (PC) of acetylcholine required to cause 5, 10, 20 and 30 fold increases from baseline R_L, the differences compared with nedocromil sodium treatment were even more striking as demonstrated in Table 1.

Eosinophilic infiltration

Repeated ovalbumin challenge increased the numbers of eosinophils per mm^2 within the epithelium of airways when compared with unchallenged control animals. The values and regression lines for numbers of eosinophils per mm^2 versus size of airway as depicted by the luminal border (Pi) are shown in Figure 3. Consistent with our previous findings (Ishida *et al.*, 1989), more eosinophils per area were observed in larger airways. Nedocromil sodium pretreatment reduced the eosinophil numbers to values intermediate between the ovalbumin only and control groups. The differences in numbers of eosinophils for the ovalbumin- and nedocromil sodium-treated groups are shown in Figure 4. It can be appreciated that there is significant overlap in the larger airways but not in the smaller airways as depicted in Figure 4b which shows the difference in numbers of eosinophils per airway size.

Discussion

In this study, we demonstrated that nedocromil sodium not only inhibited acute antigen-induced bronchoconstriction, but also inhibited both the development of airway hyperresponsiveness and the degree of airway epithelial eosinophilia following repeated antigenic challenge. Our findings of inhibition by nedocromil sodium of antigen-induced acute bronchoconstriction were anticipated in that similar results have been demonstrated in guinea-pigs (Hutson *et al.*, 1988), sheep (Abraham *et al.*, 1987), monkeys (Eady *et al.*, 1985) and man (reviewed by Thomson, 1989). The most likely explanation for this effect of the compound is inhibition of mast cell mediator release. However, the effectiveness of nedocromil sodium upon acute bronchoconstrictor responses induced by many other stimuli including sulphur dioxide (Altounyan *et al.*, 1986), adenosine (Crimi *et al.*, 1987; Phillips *et al.*, 1989), bradykinin (Fuller *et al.*, 1989) and neurokinin A (Joos *et al.*, 1989) argue for additional activities such as altered inflammatory cell infiltration or function as well as neural function.

Our finding of decreased airway hyperresponsiveness following multiple antigen challenges in nedocromil sodium-treated animals is in keeping with limited clinical data suggesting that airway hyperresponsiveness may be altered by longer term treatment with nedocromil sodium. This drug decreased airway responsiveness in grass pollen-sensitive asthmatics during the pollen season (Dorward *et al.*, 1986), and in a more recent study was demonstrated to decrease airway responsiveness in a group of nonatopic asthmatic subjects (Bel *et al.*, 1990).

The mechanism by which nedocromil sodium decreases airway responsiveness in these settings is unclear. There appears to be a distinct possibility that it has effects on both the inflammatory cell population and neuropeptide-induced effects. A role for neuropeptides in asthma has been suggested by other investigators (Lundberg *et al.*, 1985; Barnes, 1986). Recent evaluation in our laboratory (Matsuse *et al.*, 1990) has demonstrated that neuropeptide depletion of guinea-pigs by capsaicin treatment following sensitization to ovalbumin inhibits the subsequent airway hyperresponsiveness induced by repeated allergen challenge. In our study, capsaicin treatment failed to inhibit the accumulation of the eosinophils within airways, suggesting that if these eosinophils are critical in the development of airway hyperresponsiveness, their effects are mediated by neuropeptides.

The parallel changes in lung eosinophil infiltration and airway hyperresponsiveness obtained with nedocromil sodium are of interest, since two other interventions, namely capsaicin (Matsuse *et al.*, 1990) and platelet-activating factor antagonists (Ishida *et al.*, 1990), inhibited airway hyperresponsiveness without changing eosinophil numbers. Although in all the groups there were more eosinophils per epithelial area in the larger airways, perhaps reflecting a response to general inhalant insults, the greatest enhancement by allergen and inhibition by nedocromil sodium occurred in small airways, suggesting inflammatory changes at these sites may be of greater physiological significance. If eosinophils are critical for the development of airway hyperresponsiveness, nedocromil sodium may prevent hyperresponsiveness by inhibiting eosinophil recruitment or localization. Such effects could conceivably occur by inhibition of a mast cell eosinophil chemotactic factor or by inhibiting the release of a specific cytokine such as IL-3, IL-5 or GM-CSF, all of which have been shown to enhance longevity of eosinophils in culture as well as to increase mediator release from eosinophils (Sanderson *et al.*, 1985; Yamaguchi *et al.*, 1988; Rothenberg *et al.*, 1988). An additional effect of nedocromil sodium may be to decrease the release of mediators from eosinophils present in the airways for it has been shown to have this effect upon eosinophils *in vitro* (Spry *et al.*, 1986).

In summary, we have demonstrated that pretreatment with nebulized nedocromil sodium inhibits both the airway eosinophilia and airway hyperresponsiveness produced with repeated antigen challenge in guinea-pigs. Whether its effects on these two parameters represent a common mechanism of action, or whether the effect of the drug in decreasing airway eosinophils accounts for the reduction in airway responsiveness, remains to be determined.

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Block of ATP-regulated potassium channels by phentolamine and other α-adrenoceptor antagonists

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1 The patch clamp technique has been used to characterize the effects of phentolamine, an unselective blocker of α_1- and α_2-adrenoceptors, on the electrical activity of isolated RINm5F insulin-secreting cells and the gating of ATP-regulated potassium (K^+_{ATP}) channels.

2 Current-clamp experiments carried out by use of both conventional whole-cell recordings and nystatin-perforated cells, have demonstrated that phentolamine (5–20 μM) in the complete absence of α-adrenoceptor agonists, caused a sharp depolarization of the cell membrane from approximately −66 mV to −42 mV. This depolarization was associated with the generation of calcium action potential-like spikes. In the continued presence of phentolamine, diazoxide (100 μM) reversed these effects by causing a hyperpolarization of the cell, thereby preventing Ca^{2+} spikes.

3 Unitary current events from K^+_{ATP} channels were recorded from both outside-out membrane patches and saponin permeabilized or open-cells. When added to either the inside or the outside of the plasma membrane, phentolamine (0.1–100 μM) blocked openings from these channels. The effects of phentolamine were rapid, sustained and fully reversible. Phentolamine was apparently a more effective blocker of channels from the inside than the outside of the membrane.

4 The K_I value, corresponding to 50% inhibition of channels was estimated to be approximately 0.7 μM when phentolamine was added to the inside of the membrane and the Hill coefficient approximately 1.

5 Yohimbine (1–10 μM) and the chemically 2-substituted imidazoline α-adrenoceptor antagonists, antazoline (25 μM) and tolazoline (25 μM) were also found to block K^+_{ATP} channels in isolated patches of membrane.

6 In conclusion the present study demonstrates that phentolamine and other imidazoline adrenoceptor antagonists have effects upon ATP-sensitive K^+ channels that are not associated with stimulation of the adrenoceptor.

Keywords: Patch-clamp; K^+_{ATP} channels; adrenoceptor; RINm5F; insulin secreting cell; yohimbine; imidazoline

Introduction

Phentolamine, which blocks both α_1- and α_2-adrenoceptors, has effects upon insulin-secreting cells that cannot only be attributable to antagonism of the adrenoceptor. In the absence of any exogenous agonist, phentolamine initiates insulin secretion both *in vivo* (Lundquist, 1972) and *in vitro* (Malaisse *et al.*, 1967; Efendic *et al.*, 1975; Smith & Furman, 1988; Chan *et al.*, 1988; Schulz & Hasselblatt, 1989; Garrino & Henquin, 1990). In addition, phentolamine has been found to alleviate the diazoxide-induced inhibition of insulin secretion and the enhanced rate of $^{86}Rb^+$ efflux from pre-loaded islets (Burr *et al.*, 1971; Henquin *et al.*, 1982), effects that can be explained by the recent observations by Plant & Henquin (1990) that phentolamine inhibits whole-cell K^+ currents in isolated cells. Closure of adenosine 5'-triphosphate (ATP)-sensitive potassium (K^+_{ATP}) channels, initiates a depolarization of the cell membrane, resulting in the opening of voltage-operated Ca-channels and a subsequent rise in the cytosolic Ca^{2+} concentration, the key internal regulator of insulin secretion (as reviewed by Petersen & Findlay, 1987; Ashcroft, 1988; Ashcroft & Ashcroft, 1990).

In the present study the actions of phentolamine have been investigated under current clamp conditions (whole-cell and nystatin perforated cells) and at the single channel current level. The results indicate that phentolamine has effects upon individual cells that are similar to those of glucose by causing a marked inhibition of K^+_{ATP} channels, a depolarization of the cell membrane and the initiation of Ca^{2+} spike potentials.

Methods

Cell isolation and maintenance

All experiments were carried out on the clonal insulin-secreting cell line RINm5F, maintained as previously described (Dunne *et al.*, 1986).

Media

The standard extracellular Na^+-rich solution used throughout these experiments, contained (mM): NaCl 140, KCl 4.7, $MgCl_2$ 1.3, $CaCl_2$ 2.5 and HEPES 10. The pH was adjusted to 7.2 with NaOH. The standard intracellular K^+-rich solution contained (mM): KCl 140, NaCl 10, $MgCl_2$ 1.13, HEPES 10, EGTA 1 and ATP 1. No $CaCl_2$ was added and the pH was adjusted to 7.2 with KOH. Stock solutions of diazoxide (Glaxo Research Limited, UK), antazoline, tolazoline and nystatin (Sigma, UK) were prepared in dimethylsulphoxide (DMSO). The maximal concentrations of DMSO (1%) used in the final solutions for experiments had no direct effect upon K^+ channels (Dunne *et al.*, 1987). Phentolamine mesylate was obtained from Ciba-Geigy, UK. The osmolality of all solutions was 290 ± 5 mosmol kg^{-1}. All experiments were carried out at room temperature, 22–25°C.

Patch-clamp experiments

Patch-clamp recordings from excised outside-out membrane patches, nystatin perforated and whole cells (Hamill *et al.*, 1981) were made with the K^+-rich solution in the pipette and the Na^+-rich solution in the bath. For perforated patch experiments ATP was omitted from the K^+-rich solution and nystatin (20 μg ml^{-1}) added. Permeabilized or open-cell experiments (Dunne *et al.*, 1986), in which the plasma membrane was perforated by saponin (0.05%) (Sigma, UK) in contact with the membrane outside the area from which the single-channel current recording was made, were performed with the K^+-rich solution (without ATP) in the bath and the Na^+-rich solution (without $CaCl_2$) in the pipette. Patch-clamp pipettes (Type 101 PB, Ceebee Glass, Denmark) were found to have a final resistance of between 5 and 10 MΩ when filled.

Exchange of the bathing solution from control to test, was achieved manually under visual control by a series of outlet pipes (Dunne *et al.*, 1988).

All current and voltage recordings have been either photographed directly from the pen recording trace, filtered at 20 Hz (low pass) or digitized and then plotted by use of a CED software package (Cambridge, UK). Upward deflections represent outward current flow (i.e. from the inside to the outside of the membrane patch). Changes in potassium channel open-state probability (P) have been quantified from pre-recorded stretches of data lasting between 15 and 20 s, and are expressed as a fraction of the pre-control value (P_c) (Dunne, 1989). Analysis has been performed with a CED 1401 interface and a software package supplied by CED (Cambridge, UK).

Results

Nystatin-perforated and conventional whole-cells have been used to investigate the effects of phentolamine on the electrical activity of individual cells. Under current-clamp conditions the average resting membrane potential was found to be -67 ± 4 mV (mean $\pm$ s.e.mean, $n = 7$ separate cells). When challenged with phentolamine (added directly to the bath) over the range of 5–20 μM, the cells underwent a rapid depolarization, which was maximal within 45 s of stimulation, found on average to be 24 ± 2 mV ($n = 7/7$ applications) and was associated with the persistent generation of Ca^{2+} action potential spikes. In the continued presence of phentolamine, 100 μM diazoxide reversed the effects of the drug by causing a marked repolarization of the membrane which led to the termination of spike potentials ($n = 6$ separate cells) (Figure 1). The maximal effect of diazoxide was seen between 8 and 22 s following its addition to the bath.

Effects of phentolamine on unitary current events from ATP-sensitive potassium channels have been studied with both permeabilized or open-cells and excised outside-out patches. A typical open-cell experiment is shown in Figure 2. The record begins 25 s after 0.05% saponin was added to the bath solution. Dialysis of the cell leads to the wash-out of cytosolic ATP and the subsequent activation of K^+_{ATP} channels, as has been previously described (Dunne *et al.*, 1986). At the points indicated ATP (1 mM) and phentolamine (10 μM) were individually added to the bathing solution, which, under these conditions, is now in contact with the inside of the plasma membrane. Both ATP and phentolamine inhibited the K^+_{ATP} channels, reducing the relative open-state probability

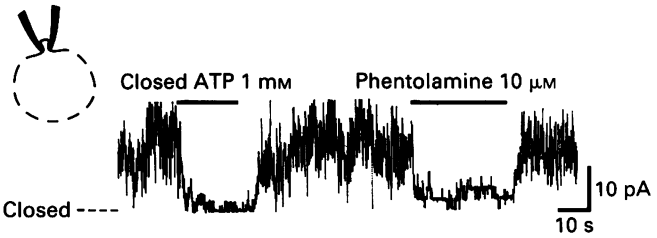

Figure 2 Inhibition of ATP-sensitive potassium channels by phentolamine. The current record shows how both ATP (1 mM) and phentolamine (10 μM) inhibit K^+_{ATP} channels in a RINm5F open-cell. The current trace starts 25 s after permeabilization of the cell was complete.

(P/P_c) from 1 to 0.16 and 0.31, respectively. The actions of both ATP and phentolamine were fully reversible upon removal of the compounds from the bath; $P/P_c = 1.17$ and 1.05, respectively. Over the range 0.1 to 100 μM, phentolamine was added to a total of 10 separate open-cells. In one of these it was possible to complete the full range of phentolamine concentrations several times, and the concentration-response relationship for this patch has been shown in Figure 3. The K_I value of 50% channel inhibition was estimated to be approximately 0.7 μM, and the data fitted by a Hill coefficient of 0.95, according to the equation:

$$\frac{P}{P_c} = \frac{1}{1 + ([x]/K_i)^h} \quad (1)$$

where K_i is the concentration of phentolamine that produces half-maximal inhibition of K^+_{ATP} channels ($P/P_c = 0.5$), [x] is the concentration of the inhibitor and h is the slope parameter (Hill coefficient).

Inhibition of K^+_{ATP} channels by 0.1 μM phentolamine was seen on a further 3/4 occasions in 4 separate cells; 1 μM phentolamine on 6/6 occasions in 4 separate cells; 10 μM phentolamine, $n = 5/6$ in 6 separate cells and 100 μM closed channels in 4 separate cells, $n = 4/4$.

The effects of 5 μM phentolamine on K^+_{ATP} channels when added to the outside of the membrane is shown in Figure 4. The record was obtained from an excised outside-out membrane patch, recorded with 1 mM ATP on the cytoplasmic face of the membrane. Phentolamine reversibly inhibited channels reducing P/P_c from 1 to 0.26 in the record shown, and to an average value of 0.45 ± 0.1 in 4 separate patches.

Channel blockade from either the inside or the outside of the membrane was rapid, readily reversible and was not

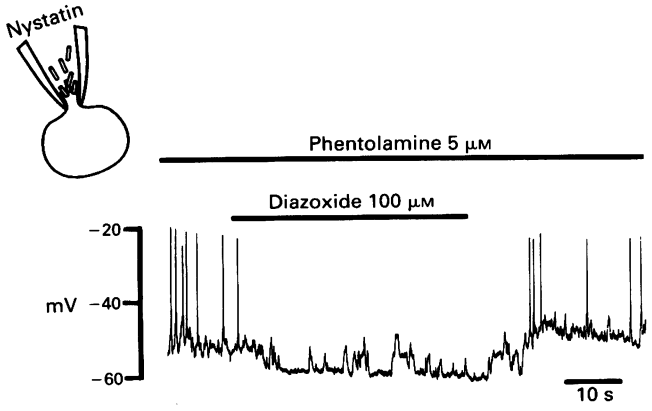

Figure 1 Effects of diazoxide on the phentolamine-induced electrical activity of an isolated insulin-secreting cell. The continuous voltage record, which was obtained with a nystatin-perforated cell (current-clamp mode), illustrates that in the continued presence of phentolamine (5 μM), diazoxide (100 μM) reverses the effects of the drug on the membrane potential.

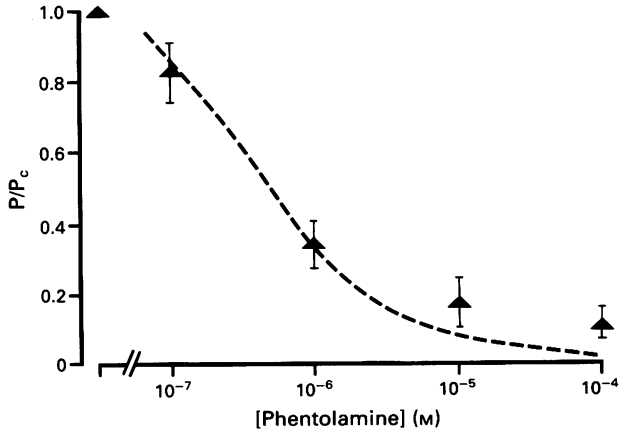

Figure 3 Concentration-dependent block of K^+_{ATP} channels by phentolamine. The concentration-response curve was obtained from a single open-cell exposed to phentolamine over the range 0.1 to 100 μM. Each point represents the mean (s.e.mean shown by vertical bars) of between 4 and 6 additions of each of the concentrations of phentolamine to the patch. The curve is drawn to equation 1 (see Results section), with a Hill coefficient of 0.95 and a K_i value of 0.7 μM.

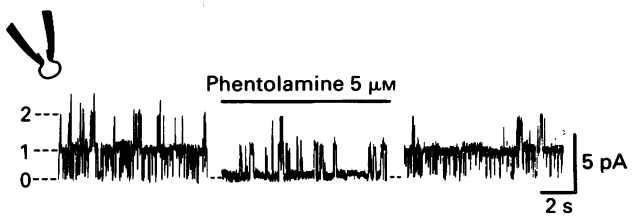

Figure 4 Block of K^+_{ATP} channels by phentolamine applied to the outside of the membrane. The record was taken from an excised outside-out path, recorded with 1 mM ATP on the cytoplasmic face of the membrane. The scale bar on the left-hand side of the current trace corresponds to the number of coincident K^+ channel openings.

associated with any significant reduction in the amplitude of the single channel current event.

Inhibition of ATP-sensitive potassium channels (open-cell records) was also brought about by the α-adrenoceptor antagonists antazoline (25 μM) which reduced P/P_c from 1 to 0.28 ± 0.08 ($n = 4$), tolazoline (25 μM) $P/P_c = 0.49 \pm 0.06$ ($n = 4$) see Figure 5, and by yohimbine (10 μM) $P/P_c = 0.19 \pm 0.09$ ($n = 3$). The actions of antazoline, tolazoline and yohimbine were not associated with any marked decrease in the amplitude of the unitary current events.

Discussion

The present work illustrates that phentolamine is a highly effective blocker of ATP-sensitive potassium channels in insulin-secreting cells, which agrees with the data of Plant & Henquin (1990) who have recently shown that the α-adrenoceptor antagonist inhibits whole-cell K^+ currents and ^{86}Rb efflux from pre-loaded islets. Phentolamine-evoked closure of K^+_{ATP} channels explains how the drug is able to initiate changes in the cell electrical activity similar to those brought about by either glucose or glyceraldehyde in these cells (Dunne, 1990) by promoting a depolarization of the membrane and the generation of Ca^{2+} spike potentials. Figure 1 shows that diazoxide — a specific activator of K^+_{ATP} channels in insulin-secreting cells (Trube *et al.*, 1986; Dunne *et al.*, 1987; Dunne, 1989), reverses the effects of phentolamine on the cell electrical activity and this fits well with the previous observations that phentolamine counteracts the actions of diazoxide on ^{86}Rb efflux and insulin-secretion (Burr *et al.*, 1971; Henquin *et al.*, 1982).

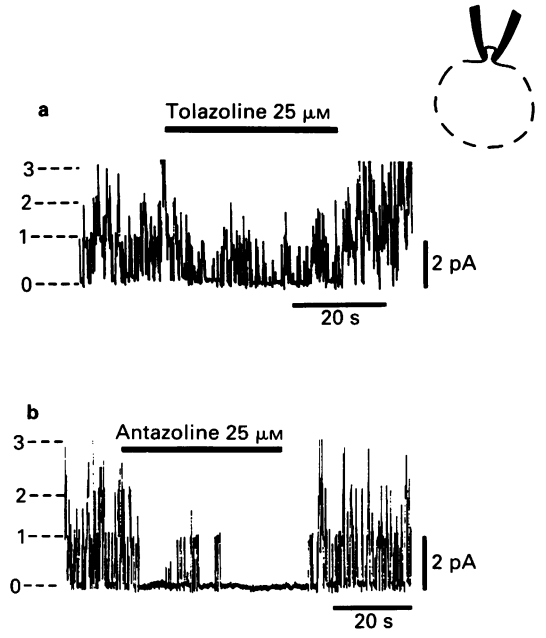

Figure 5 Blockade of K^+_{ATP} channels by tolazoline (a) and antazoline (b); both records come from separate open-cell patches. The scale bar on the left-hand side of each current trace corresponds to the number of coincident K^+ channel openings.

By use of a combination of excised outside-out patches and permeabilized cells, it has been demonstrated that phentolamine is an effective blocker of K^+_{ATP} channels when it is added directly to either the inside or the outside of the plasma membrane. The concentration-dependent block of K^+ channels in open-cells was half maximal at 0.7 μM, fitted by a Hill coefficient of 0.95 and not associated with significant effects upon the amplitude of the single-channel current event. The efficacy of channel blockade was found to be more potent when phentolamine was applied internally, since 5 μM phentolamine in outside-out patches reduced the relative open-state permeability of channels by approximately 55% vs 80% in open-cells. This finding may indicate that the binding site for phentolamine is located on the inner plane of the plasma membrane. Indeed if this is the case it may explain why using the whole-cell recording configuration, where it is harder to completely 'wash' the membrane, Plant & Henquin (1990) reported the effects of phentolamine to be poorly reversible. In my experiments the actions of phentolamine upon whole-cells and nystatin-perforated cells were readily reversible, but the relative concentrations of the drug were very much lower than those used by Plant & Henquin (1990).

The K_I value corresponding to 50% closure of K^+_{ATP} channels, was estimated to be approximately 0.7 μM, which indicates that phentolamine is as effective a blocker of K^+_{ATP} channels as tolbutamide, $K_I = 3$–18 μM (for review see Ashcroft & Ashcroft, 1990). The efficacy of channel inhibition was apparently not enhanced by the presence of cytosolic ATP, since in outside-out patches with 1 mM ATP in the pipette solution, phentolamine was less efficient at closing the channels than in open-cell experiments, which were carried out in the complete absence of ATP. One may speculate that, as with tolbutamide (Zunkler *et al.*, 1988), the potency of the drug may be further enhanced by the presence of internal ADP.

There can be little doubt that imidazoline-based adrenoceptor antagonists bind to sites other than the adrenoceptor (Michel & Insel, 1989). By analogy with the effects of the sulphonylureas on K^+_{ATP} channels in insulin-secreting cells (Ashcroft & Ashcroft, 1990), it is not yet clear from these preliminary studies, whether one of these sites, an 'imidazoline receptor', is part of the K^+_{ATP} channel or a separate protein. There is, however, firm evidence to suggest that the two are very closely associated, since all the imidazoline α-adrenoceptor antagonists so far tested, block K^+_{ATP} channels and initiate insulin secretion; phentolamine (as discussed), antazoline and tolazoline (Figure 5; Schultz & Hasselblatt, 1989) and efaroxan (Chan & Morgan, 1990; Dunne *et al.*, 1991). This observation can also be extended to include the substituted imidazoline idazoxan, which at low concentrations has little effect upon insulin secretion in the absence of an adrenoceptor agonist (Ostenson *et al.*, 1988; Chan & Morgan, 1990) but at higher concentrations (>100 μM) was found to reverse partially the diazoxide-induced inhibition of insulin secretion (Chan & Morgan, 1990) and block K^+_{ATP} channels (Dunne *et al.*, 1990). Yohimbine, on the other hand, is an α-adrenoceptor antagonist that does not contain an imidazoline structure but is also an efficient blocker of K^+_{ATP} channels (Plant & Henquin, 1990).

The pharmacological regulation of ATP-regulated potassium channels is complex (as reviewed by Petersen & Dunne, 1989; Quast & Cook, 1989; Ashcroft & Ashcroft, 1990). Channel blockers include the sulphonylureas, plant extracts and local anaesthetics. Since we can now add to this list certain α-adrenoceptor antagonists, there are two major

pharmacological implications of this study. Firstly, as pointed out be Plant & Henquin (1990), the results of many *in vivo* and *in vitro* studies with phentolamine and other α-adrenoceptor antagonists, may now need to be re-interpreted. Secondly, these data may provide us with a platform to develop an alternative strategy to the sulphonylurea-based drugs in the therapeutic treatment of diabetes mellitus. Such an approach would be feasible if it were possible to isolate the α-adrenoceptor blocking actions of compounds like phentolamine and efaroxan from the effects upon K^+_{ATP} channels and this may turn out to be feasible through the use possibly of pure enantiomeric forms of the drugs.

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Kinin receptors of the central nervous system of spontaneously hypertensive rats related to the pressor response to bradykinin

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1 Kinin analogues bradykinin (BK), T-kinin, Met-Lys-BK, Lys-Lys-BK, Des-Arg^9-BK with agonist activity and D-Arg^0-Hyp^3-Thi^{5,8}-D-Phe^7-BK (DAHTDBK) and Arg^9-Leu^8-BK with antagonist activity were injected into the posterior portion of the fourth cerebral ventricle of unanaesthetized rats implanted with permanent cannulae and arterial pressure was measured directly from the abdominal aorta.

2 The spontaneously hypertensive rats (SHR) were more sensitive than normotensive Wistar rats (NWR) to the pressor effect of BK and other kinin analogues. The SHR did not differ in sensitivity of the pressor response to centrally administered angiotensin II or endothelin-1.

3 Experiments with selective kinin agonists and antagonists revealed that in the SHR, as in the NWR, the receptors which mediated the central pressor response are of the BK_2 subtype.

4 Measurements of the pressor activity of kinins with different degrees of susceptibility to degradation, as well as experiments with kininase inhibitors, enalaprilat and CPP-Ala-Ala-Phe-pAB, suggest that the kininase activity in the central nervous system of SHR is reduced in comparison to that of NWR.

5 The SHR also showed increased sensitivity to BK and Lys-Lys-BK, compared with the NWR, when the kinins were injected following the administration of a mixture of the kininase inhibitors, suggesting that mechanisms other than kininase activity may play a role in the increased sensitivity of the SHR to the central pressor action of kinins.

6 An *in vivo* characterization of the kinin receptors which mediate the central pressor response showed that the interaction with DAHTDBK was reversible and of competitive nature. The pA_2 *in vivo* estimated for the kinin receptors of the SHR was 0.7 logarithm units larger than that obtained in the NWR.

7 The kinin receptors which mediate the central BK pressor effect in the SHR are of the BK_2 subtype and are similar to receptors in the NWR. The increased sensitivity to kinins in the SHR may be explained, at least in part, by their decreased kininase activity. At present it is impossible to conclude whether the difference observed in the pA_2 represents an increased affinity of the kinin receptors or can be attributed to differences amongst strains in the enzymatic degradation of the antagonist.

Keywords: Bradykinin; central nervous system; kinin receptors; pressor effect; spontaneously hypertensive rats

Introduction

The intracerebroventricular (i.c.v.) administration of bradykinin (BK) causes an increase in the mean arterial pressure (MAP) of rats (Pearson & Lang, 1969) and other species (Pearson & Lang, 1967; Lang & Pearson, 1968; Graeff *et al.*, 1969). Apparently this pressor response, which depends on a functionally intact adrenergic efference (Correa & Graeff, 1974), is mediated by kinin receptors in the medulla oblongata in or adjacent to the fourth cerebral ventricle (Lindsey *et al.*, 1988). The kinin receptors which mediate the central pressor response in the normotensive Wistar rat (NWR) are of the BK_2 subtype (Lindsey *et al.*, 1989) according to the classification of BK receptors in peripheral tissues established by Regoli & Barabé (1980).

The spontaneously hypertensive rat (SHR) showed increased sensitivity to the pressor action of i.c.v.-administered kinins in comparison to normotensive Wistar rats (NWR) (Lindsey *et al.*, 1988). The increased responsiveness observed in the SHR could be, in principle, related to alterations at any of the different levels of the neuroeffector system which determine short term changes in arterial pressure. The mechanisms which modulate the haemodynamic effect of kinins applied to the central nervous system include alterations in kininase activity, receptor sensitivity or population subtype, central sympathetic reactivity and vascular smooth muscle responsiveness. The aim of this study was to examine pharmacological aspects of the central kinin receptors which mediate the pressor response in the SHR. The classification of these receptors was obtained with the use of agonists and antagonists which interact with BK_1 and BK_2 kinin receptors (Regoli & Barabé, 1980; Vavrek & Stewart, 1985). An *in vivo* pharmacological characterization of receptors in SHR and NWR was attempted. These experiments were complemented with an investigation of the central pressor effect of angiotensin II (AII) and of kinin analogues with distinct pharmacodynamic or pharmacokinetic properties.

Methods

Four-month old female spontaneously hypertensive and normotensive Wistar rats, weighing approximately 200 *g*, with a mean arterial pressure of 148 ± 16 and 111 ± 12 mmHg, respectively, were used. The animals were anaesthetized with a mixture of pentobarbitone and chloral hydrate and permanent cannulae were placed in the posterior portion of the fourth ventricle (11.0 mm antero-posterior, 7.9 mm vertical and 0.0 mm lateral from bregma in SHR and 11.7 mm antero-posterior, 7.5 mm vertical and 0.0 mm lateral in NWR) (Paxinos & Watson, 1986). The cannulae were anchored to the skull by jeweller's screws embedded in dental acrylic cement. Following the i.c.v. surgery a polyethelene catheter PE10 connected to PE50, filled with heparinized saline, was placed in the abdominal aorta through the left femoral artery. The other end of the cannula was slipped beneath the skin and exteriorized on the back of the animal. After the surgery, the rats were individually housed in plastic cages (30 × 20 × 10 cm) which also served as recording chambers. Two days after implantation surgery the effect of centrally

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administered BK and analogues on the mean arterial blood pressure (MAP) was recorded, in the unanaesthetized and unrestrained animals, with a Narco P-1000B pressure transducer and a DMP-4B physiograph (Narco Biosystem, Houston, TX, U.S.A.). Dose-response curves for BK, TK, MLBK and LLBK were obtained by injecting 1 μl saline containing various concentrations of peptides at 30 min intervals; no more than three doses were administered to each animal. Delivery of the injected volume took slightly less than 1 s. Latency for the manifestation of the pressor response to peptides was defined as the time elapsed between the moment of the injection and the onset of the pressor effect of the ED_{50} dose of each peptide. Proper cannula placement was verified histologically in all rats by an injection of dye at the end of the experiment. The rat brains were excised and placed in 10% formaldehyde for two weeks before the histology. The lack of diffusion of the dye into the expected ventricular space was the criterion for excluding an animal. Regression lines obtained for the linear parts of dose-response curves were compared for critical differences following covariance analyses and tests for regression, linearity and parallelism (Snedecor & Cochran, 1980). The ED_{50} values and respective confidence intervals were estimated by a linear calibration method (Snedecor & Cochran, 1980). For differences between independent means, Student's t test was used, preceded by analysis of variance in the case of multiple comparisons.

The peptides bradykinin (BK), Met-Lys-BK (MLBK), T-kinin (Ile-Ser-BK, TK) Lys-Lys-BK (LLBK), des-Arg^9-BK (DABK), des-Arg^9-Leu^8-BK (DALBK), angiotensin II (AII) are synthetic products made in this laboratory. D-Arg-[Hyp^3, Thi^{5,8}-D-Phe^7]-BK (DAHTDBK) and Thi^{5,8}-D-Phe^7-BK (TDBK) were kindly supplied by Dr J.M. Stewart from the University of Colorado (Denver, CO, U.S.A.) and endothelin-1 was purchased from Sigma Chemical Co. The peptidase inhibitor, N-[1(RS)-carboxy-3-phenyl-propyl]-Ala-Ala-Phe-pAB (CPP-Ala-Ala-Phe-pAB) was a kind gift from Dr M. Orlowski, Mount Sinai School of Medicine, University, New York, NY, U.S.A., and enalaprilat was kindly provided by Merck Sharp & Dohme.

Results

Effect of kinin agonists on arterial pressure of SHR and NWR

Bradykinin produced a pressor response in SHR, typically lasting several minutes following the injection of the peptide into the fourth cerebral ventricle. The effect, which led to an increase of the MAP of up to 60 mmHg in some animals, was similar to the response observed in the NWR. However, the SHR responded to doses of 1 pmol or less whereas the normotensive animals responded only to doses 4 to 8 times larger. Both NWR and SHR presented pressor responses to the kinin analogues TK, MLBK or LLBK. Full dose-response curves were obtained for each analogue (Figure 1). The potency ratios, estimated by comparison of the ED_{50} values obtained in NWR and SHR (Table 1), show that BK was seven times more potent in the SHR, TK eight times as potent, LLBK four times and MLBK three times as potent as in NWR. The parameters regarding the pressor effect of the kinins injected into the fourth ventricle are shown in Table 1.

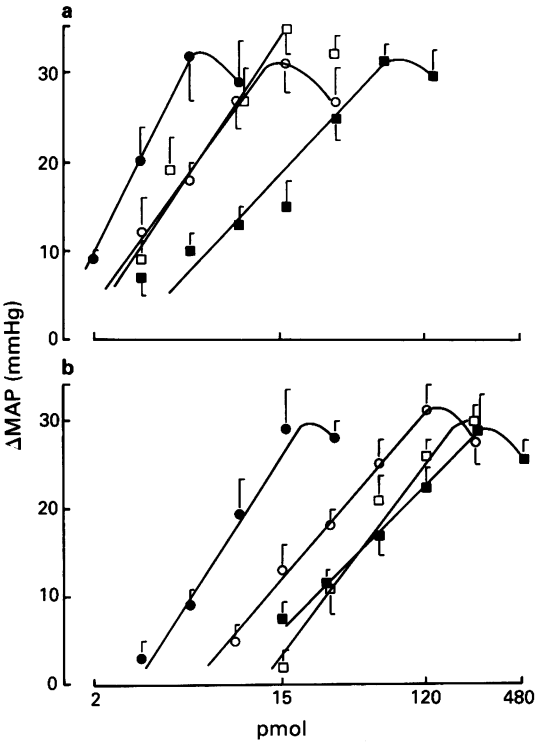

Figure 1 Log dose-response curves for the effect of kinins injected into the fourth cerebral ventricle upon the mean arterial pressure (MAP) of SHR (a) and NWR (b). The curves for bradykinin (BK) (○), Lys-Lys-BK (LLBK) (●), Met-Lys-BK (MLBK) (■) and T-kinin (□) in the SHR differed significantly ($P \leq 0.001$) from those in the NWR. The linear portion of the curves were parallel with the exception of the curve for MLBK which was not parallel to that for LLBK. Each point represents the mean of 10–15 determinations and the s.e.mean are indicated by vertical bars.

Table 1 Pressor effect of kinins injected into the fourth ventricle of SHR and NWR

	ED_{50} (pmol)		Latency (s)		Maximal effect (mmHg)		Duration (min)	
Peptide	*SHR*	*NWR*	*SHR*	*NWR*	*SHR*	*NWR*	*SHR*	*NWR*
LLBK	1, 5*† (0.2–8)	6† (2–19)	11 ± 8*§	16 ± 9§	32 ± 16	29 ± 11	5 ± 4	5 ± 4
BK	3 (0.7–14)	22 (1–238)	9 ± 8	16 ± 10	31 ± 14	31 ± 15	4 ± 4	4 ± 3
TK	4, 5 (0.7–25)	43 (7–240)	13 ± 8	20 ± 9	35 ± 14	30 ± 9	2 ± 2†	3 ± 3
MLBK	14† (1–87)	45 (8–240)	14 ± 8	18 ± 8	31 ± 9	29 ± 11	6 ± 5	8 ± 8

BK, bradykinin; LLBK, Lys-Lys-BK; TK, T-kinin; MLBK, Met-Lys-BK.
The ED_{50} values and respective 95% confidence limits (shown in parentheses) were calculated from linear portions of the dose-response curves (Figure 1).
The values for latency, maximal effect and duration represent the mean ± s.d. of at least twenty determinations.
* All values of the column differ ($P < 0.05$) from the respective values obtained in the NWR.
† Differs significantly ($P < 0.05$) from all values of the same column.
§ The latency values for BK and LLBK differ ($P < 0.05$) from those for TK and MLBK.

Among the kinins studied, LLBK was the most potent in the SHR, BK and TK ranked after LLBK and MLBK was the least potent analogue. Almost the same order was followed in the NWR, with the difference that BK was more potent than TK and MLBK, which were equipotent in normotensive animals. The mean time elapsed for the onset of the pressor effect in the hypertensive animals ranged from 9 to 14 seconds, depending on the kinin. The shortest latencies were observed for BK and LLBK whereas longer latencies were observed for TK and MLBK (Table 1). The latency for manifestation of the pressor effect in the NWR showed a significant correlation ($r = 0.98$) to the values obtained in the SHR. However, in the normotensive rats the latencies observed for all the kinins studied were approximately 50% longer than in their hypertensive counterparts. No difference was observed between NWR and SHR in the duration of the pressor response to kinins. The effects of BK and TK had shorter durations while MLBK had the longest lasting effect in both strains of rats. An inverse correlation ($r = 0.97$) was observed between the potency ratios of kinins in NWR and SHR and the duration of the pressor response. The mean arterial maximal effect of approximately 30 mmHg was the same for all kinins in both SHR and NWR (Figure 1).

Effects of angiotensin II and endothelin-1 on arterial pressure

Angiotensin II injected into the fourth ventricle of SHR or NWR produced an increase in the MAP with the same maximal effect (33 ± 11 mmHg for SHR and 31 ± 17 mmHg for NWR) as BK. However, AII was 50 and 250 times less potent than BK in the NWR and in the SHR, respectively (Figure 2). The latency for the onset of the pressor response was significantly shorter for AII than for any of the kinins (Table 2). The pressor effect of AII lasted from 8 to 10 min and was significantly longer than that observed for the kinins with the exception of MLBK in the NWR. Angiotensin II was equipotent in the SHR and NWR and there was no significant difference in the duration of the pressor effect, although the SHR showed a significantly shorter latency for the manifestation of that effect. Endothelin-1 like AII and BK, produced a pressor response in the NWR and SHR. Endothelin-1 was equipotent in SHR and NWR ($ED_{50} = 5.0$ and 5.2 pmol respectively) and the maximal effect of 31 ± 8 and 29 ± 11 mmHg was achieved with the dose of 10 pmol in SHR and NWR respectively.

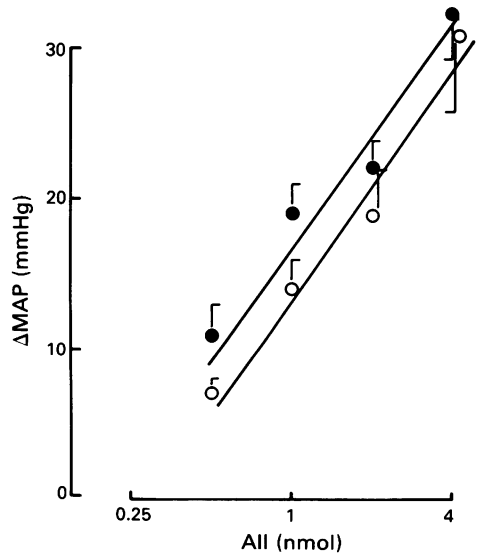

Figure 2 Linear portions of the log dose-response curves for the systemic pressor effect of angiotensin II (AII) injected into the fourth cerebral ventricle of NWR (○) and SHR (●) rats. The linear portion of the curves were parallel and did not differ significantly from each other ($P > 0.2$). Each point represents the mean of at least 8 observations and the s.e.mean are indicated by vertical bars.

Table 2 Effect of angiotensin II injected into the fourth cerebral ventricle of SHR and NWR

	ED_{50} (pmol)	*Latency* (s)	*Maximal effect* (mmHg)	*Duration of response* (min)
SHR	830 (150–4283)	1 ± 1*	33 ± 11	7 ± 5
NWR	1137 (196–6383)	4 ± 3	31 ± 17	8 ± 6

The ED_{50} values and 95% confidence limits (in parentheses) were calculated from the log-dose response curves of Figure 2. The values for latency, maximal effect and duration of the pressor response are represented by the mean ± s.d. of at least 20 animals.
* Significantly different ($P < 0.01$) from NWR.

Effect of kininase inhibitors on the response to kinins

The enzyme inhibitors were injected into the fourth cerebral ventricle dissolved in 2 μl of saline 10 min before the administration of kinins. Preliminary experiments showed that maximal potentiation of the BK pressor effect was observed from 5 to 15 min following the administration of the inhibitors. Pretreatment with a mixture of the kininase inhibitors enalaprilat (0.6 μmol) and CPP-Ala-Ala-Phe-pAB (2.4 μmol), injected into the fourth ventricle 10 min before the administration of the kinins, potentiated the effect of BK in the NWR and the SHR (Table 3). The pressor effect of LLBK was not altered by pretreatment with the enzyme inhibitors. Interpolation of the effect in the presence or absence of kininase inhibitors on the dose-response curves from Figure 1, revealed that BK was potentiated approximately fivefold in the NWR and threefold in the SHR. In the presence of the kininase inhibitors the SHR continued to show increased sensitivity to the pressor effects of BK or LLBK.

Effect of agonists and antagonists on the central pressor response to bradykinin in the SHR

For the antagonism studies, the analogues were mixed with BK in the appropriate concentrations so that 1 μl contained the desired amount of agonist and antagonist. Preliminary experiments showed that the inhibitor DAHTDBK rapidly blocks the BK receptors. The intracerebroventricular injection of the antagonists 5 or 10 min before administration of BK did

Table 3 Effect of the kininase inhibitors enalaprilat (0.6 μmol) and CPP-Ala-Ala-Phe-pAB (2.4 μmol) on the pressor effect (MAP) of bradykinin (BK) or Lys-Lys-BK (LLBK) injected into the fourth cerebral ventricle of NWR and SHR

	Control MAP (mmHg)	*Kininase inhibitors MAP* (mmHg)
NWR (BK 8 pmol)	4 ± 8	20 ± 13*
SHR (BK 2 pmol)	12 ± 9	29 ± 9*
NWR (LLBK 2 pmol)	6 ± 5	9 ± 7
SHR (LLBK 1 pmol)	7 ± 7	7 ± 7

The values represent the mean ± s.d. of 20 animals.
The kininase inhibitors, dissolved in saline, were injected into the fourth cerebral ventricle 10 min before injection of BK or LLBK.
Differs significantly from controls ($P < 0.001$, paired t test).

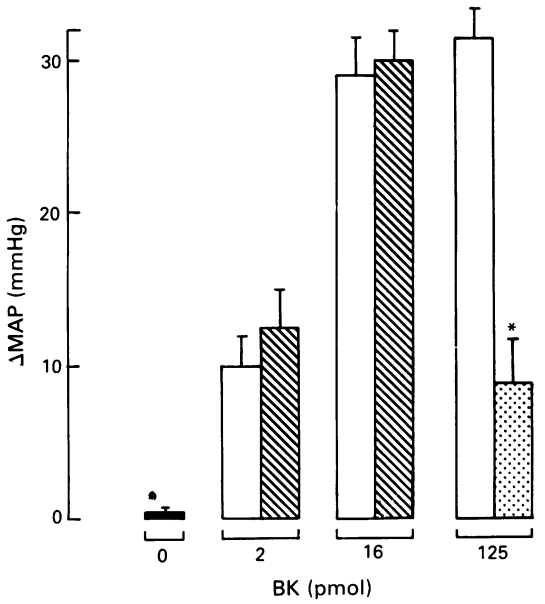

Figure 3 Effect of 12 nmol of the BK_1 receptor antagonist des-Arg^9-Leu^8-bradykinin (DALBK, hatched columns) or 12 nmol of the BK_2 antagonist Thi^{5,8}-D-Phe^7-BK (TDBK, stippled column) on the response to 60 or 125 pmol of bradykinin (BK, open columns). The solid column shows the effect of DALBK alone. Each column is the mean of 5 to 10 determinations and the s.e.mean are indicated by vertical bars.

not enhance inhibition. Thirty minutes after DAHTDBK, no inhibition of the pressor effect could be observed. No change in MAP was observed after the administration of the BK_1 bradykinin agonist DABK in the dose range of 8–11,500 pmol

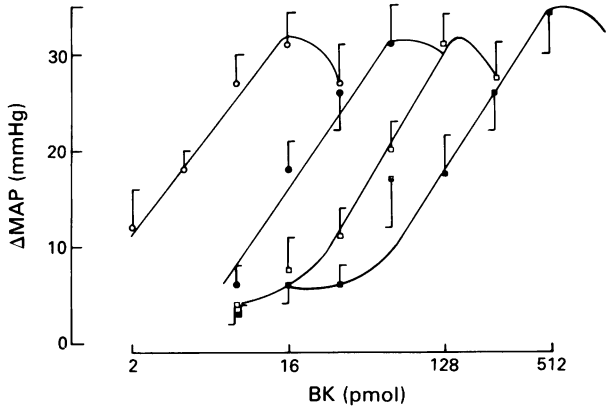

Figure 4 Log dose-response curves obtained for bradykinin (BK) on the mean arterial pressure (ΔMAP) alone (○) and in the presence of different doses (●, 0.08; □, 0.2; ■, 0.8 nmol) of D-Arg-[Hyp^3, Thi^{5,8}-D-Phe^7]-BK. The regression lines were parallel and differed significantly from each other ($P < 0.01$). Each point is the mean of determinations carried out in 10 to 15 animals and the s.e.mean are indicated by vertical bars.

Table 4 Inhibition of the pressor effect of Lys-Lys-bradykinin (LLBK) by the antagonist D-Arg^0-Hyp^3-Thi^{5,8}-D-Phe^7-BK (DAHTDBK) in SHR and NWR

	LLBK (pmol)	*DAHTDBK* (pmol)	*Antagonist/agonist ratio* (DAHTDBK/LLBK)
SHR	8	30	3, 7
NWR	16	100	6, 3

Dose of LLBK used to cause a mean pressor effect of 27 to 29 mmHg in SHR and NWR.
Dose of antagonist which caused a mean reduction of 50% in the maximal pressor effect of LLBK.

($n = 8$ per dose). The BK_1 antagonist DALBK, in doses up to 11,500 pmol, did not alter blood pressure in the SHR nor did DALBK, administered i.c.v. simultaneously with the agonist, interfere with the pressor response to BK (Figure 3). On the other hand, the BK_2 antagonist TDBK, at a molar ratio of 10:1 with respect to the agonist, produced a 70% inhibition of the effect of 125 pmol BK (Figure 3). The pressor effect of BK was also inhibited by another BK_2 antagonist. Different amounts of DAHTDBK produced parallel shifts to the right of the dose-response curves for BK (Figure 4). Schild plots (Arunlakshana & Schild, 1959) of the data from Figure 4 and similar data previously obtained in normotensive animals (Lindsey *et al.*, 1989) yielded linear plots ($r = 0.98$ for SHR and $r = 0.99$ for NWR) with similar slopes (−1.01 for SHR and −1.16 for NWR). The pA_2 *in vivo* was estimated to be 10.66 for SHR and 9.91 for NWR. Another experiment, designed to estimate the potency of the antagonist in the SHR was carried out with LLBK as an agonist instead of BK. The dose of antagonist DAHTDBK sufficient to reduce by 50% the response of an equipotent dose of LLBK was estimated in both strains of rats. Table 4 shows that the agonist: antagonist ratio was 6 for the NWR and approximately 4 for the SHR.

Discussion

The spontaneously hypertensive rats are more sensitive than normotensive rats to the systemic pressor action of kinins injected into the fourth cerebral ventricle. Bradykinin, TK, MLBK and LLBK are from three to seven times as potent in the SHR than in the NWR. Another significant difference between the two strains is the shorter latency for the manifestation of the pressor response to the kinins in the hypertensive animals. At present it is difficult to ascertain whether the increased sensitivity observed in the SHR is a selective trait related to the central action of kinins on blood pressure or whether it is due to widespread alterations in the SHR including aspects of neuronal reactivity. The finding that the SHR is not more sensitive than the NWR to the pressor effect of AII or endothelin injected into the fourth cerebral ventricle indicates that the increased sensitivity would be specific for BK. There is convincing evidence that the SHR display a hyperreactivity of the sympathetic nervous system (Okamoto *et al.*, 1967; Takahashi & Buñag, 1980), and this might explain the increased pressor responses to centrally administered BK in anaesthetized and pithed SHR (Takahashi & Buñag, 1980). However the anaesthesia and deafferentation affect the sympathetic tone and sympathetic/parasympathetic balance in an unpredictable manner, rendering impossible any comparison with the present experiments.

The present data, obtained in conscious rats, suggest that pharmacodynamic and pharmacokinetic aspects of the handling of kinins in the central nervous system may play a relevant role in the increased responsiveness of the SHR to BK and analogues. The biological activity of kinins in a given system results from the interplay of affinity, efficacy and susceptibility to degradation. Molecular degradation carried out by kininases represents the most important mechanism known to reduce the biological activity of kinins, and the differences in ED_{50} ratios for each kinin in the NWR and SHR probably reflect a decreased kininase activity in the hypertensive animals. The two kinins LLBK (Roblero *et al.*, 1973; Lindsey *et al.*, 1987) and MLBK (Roblero *et al.*, 1973) which are more resistant to kininase activity showed lower potency ratios when the respective ED_{50} values for the pressor effect were compared in the NWR and SHR. The relationship between susceptibility to degradation and potency ratio between strains is bolstered by the inverse correlation which is observed between these ratios and the duration of the pressor response, a parameter which itself may represent an index of kininase activity. A number of peptidases are believed to be active in metabolizing kinins in the central nervous system. Angiotensin converting enzyme (ACE), or kininase II has been

detected in neural tissue (Yang & Neff, 1972) and cerebrospinal fluid (Wigger & Stalcup, 1978) probably as an ACE isoenzyme (Strittmatter *et al.*, 1985). Enalaprilat, in the nanomolar range, inhibits ACE activity (Shapiro & Riordan, 1984). Other enzymes, such as EC 3.4.24.15, (Orlowski *et al.*, 1983), EC 3.4.24.19 (Oliveira *et al.*, 1990) or EC 3.4.21.26 (Wilk, 1983) are also found in neuronal tissue and inactivate several neuropeptides. CPP-Ala-Ala-Phe-pAB inhibits EC 3.4.24.15 with an affinity constant (K_i) of 30 nM (Orlowski, personal communication) and the same compound was shown to inhibit 80% of the kininase activity detected in hypothalamic slices of the rat brain (McDermott *et al.*, 1987). Preliminary experiments on the inhibition of the kininase activity in the fourth cerebral ventricle carried out in our laboratory support a similar conclusion. In order to assess the contribution of kininase activity to the response to centrally administered kinins, the pressor effect of BK and LLBK was examined in rats pretreated with an association of CPP-Ala-Ala-Phe-pAB and enalaprilat. Inhibitors were injected intracerebroventricularly in doses which could reach concentrations 5 and 6 orders of magnitude greater than the respective inhibition constants, provided that no losses occurred and that the inhibitors were homogeneously distributed in cerebrospinal fluid. Pretreatment with kininase inhibitors potentiated the response to BK in the NWR approximately five times and in the SHR three times. The response to LLBK, on the other hand, was not significantly altered in either strain by the inhibitors, supporting the assumption that LLBK is relatively resistant to degradation. Notwithstanding pretreatment with kininase inhibitors, the SHR still showed increased sensitivity to the pressor action of kinins in comparison to the NWR. This result suggests that mechanisms other than reduced kininase activity may contribute to the increased sensitivity to centrally administered kinins observed in the hypertensive rats.

The BK_1 receptor agonist DABK did not cause any alteration in the mean arterial pressure following its administration to the fourth cerebral ventricle of SHR and neither did the simultaneous administration of a selective antagonist of BK_1 receptors alter the central pressor response to BK. The pressor action of BK, however, was antagonized by the BK_2 antagonists TDBK and DAHTDBK, suggesting that the receptors which mediate the central effect of BK on blood pressure are of the BK_2 subtype. Apparently the receptors which mediate the central pressor response in the SHR are of the same subtype as those which mediate the same response in the NWR (Lindsey *et al.*, 1989). The antagonist DAHTDBK interacts with the central BK_2 receptors in competitive and reversible fashion as is shown by the parallel displacements to the right of the BK dose-response curves when the agonist was injected simultaneously with different concentrations of the antagonist. The pA_2 *in vivo* (Tallarida *et al.*, 1979) estimated for the kinin receptors of the SHR was 10.66, 0.7 log units larger than the pA_2 obtained for the kinin receptors in the NWR. It has been advanced that a difference in 0.5 or more in the pA_2 values for the same antagonist can be considered preliminary evidence for different types of receptors (Furchgott, 1972). However, since the pA_2 were determined *in vivo*, without control of any of the drug removal mechanisms, it is impossible to ascertain whether the difference in pA_2 values represents an increased affinity of the SHR receptors for the antagonist or is a consequence of differences in peptidase activity in SHR and NWR. Nevertheless, differential metabolism of the antagonist amongst strains, which could lead to a difference in the estimated pA_2 is apparently not an important factor. The DAHTDBK molecule is probably very resistant to kininase activity as may be deduced by modifications introduced into the antagonist's chemical structure. Data on the duration of the antagonist's action in the fourth cerebral ventricle support the assumption that DAHTDBK is relatively resistant to degradation (Lindsey *et al.*, 1989).

BK, LLBK, MLBK and TK probably have the same intrinsic activity since the maximal responses obtained were the same for the four kinins. Significant differences were observed in the potency of BK analogues. BK, which has the greatest affinity for the BK_2 receptors in different preparations (Regoli & Barabé, 1980; Manning *et al.*, 1986), was supplanted by LLBK in potency, probably because of the latter analogues exceptional resistance to enzymatic degradation. Differences among the kinins in the latency for the manifestation of the pressor response are difficult to interpret. BK and LLBK, which have the greatest affinity for the receptor, also showed significantly shorter latencies than TK or MLBK and, curiously, the latencies observed for BK, LLBK and MLBK are in the inverse order of the relative affinity for the kinin receptor in the guinea-pig ileum (Manning *et al.*, 1986). A causal relation between the greatest affinity of the kinin receptors and the shorter latency for manifestation of the pressor response in the SHR is improbable as many other mechanisms underlie the chain of events leading to centrally mediated pressor response.

The kinin receptors which mediate the central pressor response to BK in the SHR are of the BK_2 subtype, similar to the same receptors in the NWR. Distinct mechanisms comprising a decreased kininase activity and possibly a greater affinity of the receptors for the agonists, contribute to the increased sensitivity of the SHR to the pressor effect of centrally administered kinins.

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Direct evidence for an important species difference in the mechanism of 8-OH-DPAT-induced hypothermia

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1 Parallel series of experiments were carried out in the rat and mouse in order to investigate the mechanism(s) underlying the hypothermia induced in rodents by the selective 5-HT_{1A} receptor agonist, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT).

2 In the mouse, lesioning of central 5-hydroxytryptaminergic neurones (by use of the neurotoxin, 5,7-dihydroxytryptamine; 5,7-DHT) abolished the hypothermic response to 8-OH-DPAT, and depletion of brain 5-hydroxytryptamine (5-HT) levels (with the 5-HT synthesis inhibitor, *p*-chlorophenylalanine) markedly attenuated the response in this species. These pretreatments did not significantly attenuate 8-OH-DPAT-induced hypothermia in the rat, except for a significant attenuation of the response in 5,7-DHT-lesioned rats at the top dose of 8-OH-DPAT (1.0 mg kg^{-1}, s.c.).

3 Pharmacological pretreatments which facilitate 5-HT release (selective 5-HT uptake inhibitors, precursor (5-hydroxytryptophan) loading, or fenfluramine), markedly attenuated or abolished 8-OH-DPAT-induced hypothermia in the mouse. These pretreatments generally had no significant effect on 8-OH-DPAT-induced hypothermia in the rat.

4 The selective noradrenaline uptake inhibitor, desipramine, had no effect on the hypothermic response to 8-OH-DPAT in either species. The selective dopamine uptake inhibitor, nomifensin, significantly increased the hypothermic response to 8-OH-DPAT in the mouse, but did not affect the response in the rat except at high, motor stimulant doses, when the response was attenuated.

5 These data are consistent with the hypothesis that 8-OH-DPAT-induced hypothermia is mediated by presynaptic autoreceptors in the mouse and by postsynaptic 5-HT_{1A} receptors in the rat. Preliminary data also indicate an involvement of dopamine release in the mouse but not in the rat.

Keywords: 8-OH-DPAT-induced hypothermia; species difference (mouse, rat); presynaptic 5-HT_{1A} receptors; postsynaptic 5-HT_{1A} receptors

Introduction

The selective 5-HT_{1A} receptor agonist, 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT; Hjorth *et al.*, 1982; Middlemiss & Fozard, 1983) induces a centrally-mediated hypothermia in mice and rats (Goodwin *et al.*, 1985; Hjorth, 1985; Gudelsky *et al.*, 1986; Hutson *et al.*, 1987). However, the mechanism by which 8-OH-DPAT induces this effect on thermoregulation has not been elucidated. Central 5-HT_{1A} receptors are located both presynaptically (functioning as inhibitory somatodendritic autoreceptors on 5-hydroxytryptaminergic neurones in the raphe nuclei) and postsynaptically (Dourish *et al.*, 1986). As a first step in understanding the mechanism of 8-OH-DPAT-induced hypothermia, it is important to establish whether 8-OH-DPAT either facilitates transmission along the relevant 5-hydroxytryptamine (5-HT) pathway(s) (by stimulating postsynaptic 5-HT_{1A} receptors) or inhibits transmission by stimulating somatodendritic 5-HT_{1A} autoreceptors (leading to an attenuation of 5-HT release from axonal terminals). In this context, somatodendritic autoreceptors should be clearly distinguished from inhibitory terminal autoreceptors (mediating a local negative feedback on 5-HT release into the synapse) which are of the 5-HT_{1B} or 5-HT_{1D} subtype, according to species (Engel *et al.*, 1986; Schlicker *et al.*, 1989).

In the mouse, there is good evidence that the 5-HT_{1A} receptors mediating 8-OH-DPAT-induced hypothermia are presynaptic, since presynaptic 5-HT nerve terminal lesions (produced by the selective 5-HT neurotoxin, 5,7-dihydroxytryptamine; 5,7-DHT) or 5-HT depletion (following synthesis inhibition by *p*-chlorophenylalanine; pCPA) virtually abolished the hypothermic response to 8-OH-DPAT (Goodwin *et al.*, 1985). In the rat, there is some controversy as to whether thermoregulatory 5-HT_{1A} receptors are presynaptic (Goodwin *et al.*, 1987) or postsynaptic (Hjorth, 1985; Hutson *et al.*, 1987). The latter authors found that 5-HT depletion or 5,7-DHT lesions, rather than attenuating 8-OH-DPAT-induced hypothermia, tended to enhance the response. These results indicate that the relevant 5-HT_{1A} receptors are located postsynaptically in the rat: the observed enhancement of 8-OH-DPAT-induced hypothermia would be consistent with the development of postsynaptic 5-HT_{1A} receptor supersensitivity following 5-HT depletion. However, Goodwin *et al.* (1987) found that PCPA-induced 5-HT depletion prevented 8-OH-DPAT-induced hypothermia in the rat, concluding that the 5-HT_{1A} receptors mediating this response are presynaptic.

In an attempt to resolve this controversy, we have carried out two parallel series of experiments in the mouse and rat, in which we examined the effects of facilitation or attenuation of 5-HT release on 8-OH-DPAT-induced hypothermia. In addition to the 5-HT depletion techniques (5,7-DHT; PCPA) used previously, we have examined the effects of facilitation of 5-HT release (using selective 5-HT reuptake inhibitors, precursor loading, and a 5-HT releasing agent) on 8-OH-DPAT-induced hypothermia in both species. Some of these data have been published in preliminary form (Bill & Fletcher, 1988; Bill *et al.*, 1989).

Methods

Animals

Female albino Tuck (T/O strain) mice weighing 20–27 g, and male Sprague-Dawley rats (150–250 g; Charles River) were maintained on a 12 h light:dark cycle (lights on at 08 h 00 min), and supplied with food (mice: CRM pellets; rats: 41B pellets, Labsure) and water *ad libitum*. The animals were transferred to the experimental laboratory between 11 h 00 min–

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12 h 00 min where they were housed at an ambient temperature of 20 ± 0.5°C in groups of eight (mice) or four (rats) for at least 2 h before body temperature measurement and drug administration. All experiments were performed between 13 h 00 min and 17 h 00 min.

8-OH-DPAT-induced hypothermia

Body temperatures were measured with a thermistor probe (connected to an electronic digital 2001 thermometer; Comark Electronics Limited, Sussex) inserted 2 cm into the rectum (mice) or 4 cm into the oesophagus (rat). Temperatures were measured immediately before the administration of test compounds, again immediately before 8-OH-DPAT administration, and at 15 and 30 min following 8-OH-DPAT administration. The hypothermic response to 8-OH-DPAT was measured as the maximum decrease in body temperature recorded in this latter period.

Behavioural observations

In addition to hypothermia, the overt behavioural response to 8-OH-DPAT (the 5-HT syndrome) was measured in control rats and in rats pretreated with 5,7-DHT. The intensity of the syndrome was measured 10 min after the s.c. administration of 8-OH-DPAT by assigning an arbitrary score for each component of the syndrome (see Tricklebank *et al.*, 1985), i.e. forepaw treading, headweaving, flat body posture and tremor. A score of 0–3 (corresponding to arbitrary ratings of 0 = absent, 1 = equivocal, 2 = present or 3 = severe) was assigned to each component, except tremor, which was scored as absent (0) or present (1). Therefore the maximum possible score was 10 per rat.

5,7-DHT lesions and PCPA-induced depletion

For 5,7-DHT lesioning, rats (weighing 280–320 g) were anaesthetized on day 1 with sodium pentobarbitone (Sagatal) (75 mg kg^{-1}, i.p.) and mounted in a stereotaxic frame. The skull was exposed and a hole drilled to allow a 10 μl Hamilton fixed needle syringe to be positioned for vehicle or 5,7-DHT solution (150 μg in 2.5 μl) to be infused into the third ventricle over a period of 1 min. The needle was left in position for a further minute. Coordinates from the stereotaxic brain atlas of Paxinos & Watson (1986) were used to position the tip of the needle on the midline, 2 mm posterior to the bregma, and 9.5 mm below the skull surface. On day 10, 8-OH-DPAT-induced hypothermia and syndrome were measured.

5,7-DHT (50 μg per animal) was administered intracerebroventricularly (i.c.v.) to mice in a dose volume of 5 μl/mouse by the method of Brittain (1966). The site of injection was within 1 mm of a point on the midline 2 mm rostral to a line joining the anterior bases of the ear pinnae. Injections were made with a 25 μl Hamilton syringe attached to a 26 gauge/10 mm microlance hypodermic needle (Luer type) inserted perpendicularly through the skull, and penetrating the brain to a depth of 2.5 mm below the upper surface of the skull. 8-OH-DPAT-induced hypothermia was measured on day 5 following i.c.v. administration of 5,7-DHT.

PCPA (200 mg kg^{-1}, p.o.) or vehicle were administered to rats on day 1 and day 2. On day 5, 8-OH-DPAT-induced hypothermia was measured. The same dosing schedule was used for mice, except that each dose of PCPA was 750 mg kg^{-1}, p.o.

Biochemical measurements

The dosing schedules described above were used to determine the effects of 5,7-DHT lesioning and PCPA pretreatment on mouse and rat brain levels of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), noradrenaline (NA) and dopamine. These materials were assayed by high performance liquid chromatography (h.p.l.c.) with electrochemical detection by a modification of the method of Wagner *et al.* (1982). Animals were decapitated and the brains rapidly removed. The cerebella were discarded and the remaining brains were weighed, frozen on dry ice and stored at −30°C until homogenization in 2.0 or 3.0 ml of 0.4 M perchloric acid containing 0.15% sodium metabisulphite, 0.05% disodium EDTA and 400 ng ml^{-1} of isoprenaline (internal standard). Tissue samples were then centrifuged at 20,000 *g* for 20 min at 4°C and the supernatants removed and stored at −20°C until analysis (no longer than 2 to 3 days). Aliquots (10 or 25 μl) of the supernatants were analysed by an h.p.l.c./electrochemical detection system consisting of an LKB 2150 pump, refrigerated WISP710B automatic injector, ESA Coulochem 5100A electrochemical detector and Spectra-Physics SP4200 integrator (Waters Associates, Harrow).

Drugs

Drugs were administered in a volume of 10 ml kg^{-1} (mice) or 2.5 ml kg^{-1} (rats), routinely as mg kg^{-1} of salt. PCPA and 5,7-DHT doses are expressed as mg kg^{-1} or μg of active base. Drugs administered orally were dissolved or suspended in 0.3% hydroxypropylmethylcellulose (HMPC) in distilled water, whereas parenteral injections comprised solutions in isotonic saline except for carbidopa (sonicated in saline containing 0.1 mg ml^{-1} ascorbate and a trace of Tween 80). 5,7-DHT was dissolved in sterile isotonic saline containing 0.1 mg ml^{-1} ascorbic acid.

The drugs used (and their sources) were as follows: 8-OH-DPAT hydrobromide (Wyeth Research UK), fenfluramine hydrochloride (Sigma), 5-hydroxy-L-tryptophan base (Sigma), carbidopa base (Merck, Sharp and Dohme), fluvoxamine maleate (Duphar), citalopram hydrobromide (Lundbeck), panuramine hydrochloride (Wyeth Research UK), Wy 27587 maleate (N-[[[1-[(6-fluoro-2-naphthalenyl)methyl]-4-piperidinyl]amino]carbonyl]-3-pyridine carboxamide (Wyeth Research UK)), PCPA ethyl ester hydrochloride (Sigma), 5,7-DHT creatinine sulphate (Sigma), desipramine hydrochloride (Ciba-Geigy), nomifensin hydrochloride (Wyeth Research UK).

Experimental design and statistical analysis

Animals were weighed and numbered before being allocated randomly to treatment groups, by use of random number tables (Fisher & Yates, 1957). Appropriate vehicle control groups were included in all experiments, which were performed with coded drug or vehicle solutions such that the operator was 'blind' to pretreatments. Body temperature data were analysed by the parametric one-way analysis of variance (ANOVA) before comparing pairs of treatment groups by Student's *t* test. In calculating *t* values and assessing their significance, the error mean square value and the error degrees of freedom from the ANOVA were used. Non-parametric syndrome data were analysed by the Mann-Whitney U-test. ED_{50} values (doses of 5-HT uptake inhibitors attenuating the hypothermic response to 8-OH-DPAT by 50%) and 95% confidence limits were calculated by regression analysis.

Results

8-OH-DPAT-induced hypothermia in the mouse and rat

Following subcutaneous administration, 8-OH-DPAT (0.03–3.0 mg kg^{-1}) dose-dependently reduced body temperature in the mouse and rat (Figure 1). In both species, maximal decreases in body temperature were recorded at either 15 or 30 min following 8-OH-DPAT administration (Figure 2a and b). In subsequent drug interaction studies, submaximal doses of 8-OH-DPAT were employed, i.e. 0.8 mg kg^{-1} s.c. for mice and 0.3 mg kg^{-1} s.c. for rats. Throughout the experiments described in this paper, the hypothermic responses to these

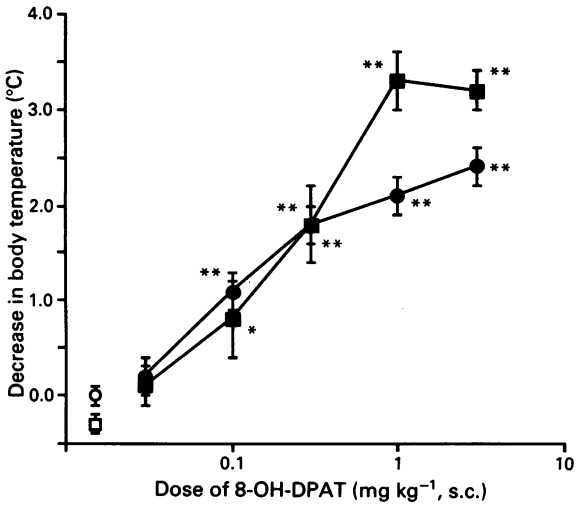

Figure 1 Dose-response relationships for 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced hypothermia in the mouse (■) and rat (●). Animals ($n = 8$) were injected s.c. with 8-OH-DPAT (0.03–3.0 mg kg^{-1}) or saline. Body temperatures were measured immediately before, and at 15, 30 and 60 min post-drug administration. The hypothermic response to 8-OH-DPAT was measured as the maximum decrease in body temperature occurring in the 30 min period post-administration of 8-OH-DPAT. Open symbols show the mean change in body temperature after s.c. injection of saline in mice (□) and rats (○). Error bars denote ± s.e.mean. * $P < 0.05$; ** $P < 0.01$ (one-way ANOVA followed by Student's t test cf. the saline-treated control groups).

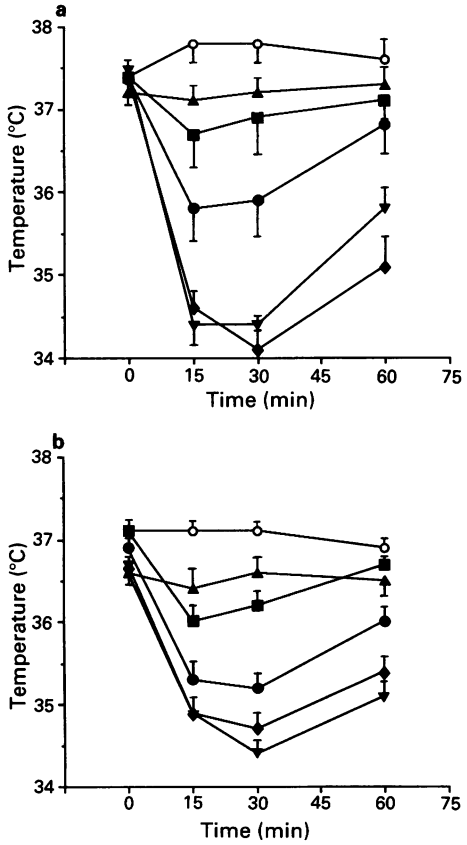

Figure 2 Time-dependent effects of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) on the body temperature of (a) mice and (b) rats. Animals ($n = 8$) were injected s.c. with either saline (○) or 0.03 (▲), 0.1 (■), 0.3 (●), 1.0 (◆) or 3.0 (▼) mg kg^{-1} of 8-OH-DPAT. Body temperatures were measured immediately before, and at 15, 30 and 60 min post-administration of 8-OH-DPAT. Maximal decreases in body temperature were recorded either 15 or 30 min post-drug injection. Error bars denote ± s.e.mean.

doses ranged from −2.0 ± 0.3 to −3.0 ± 0.4°C for mice, and from −1.6 ± 0.3 to −2.3 ± 0.3°C for rats (mean values ± s.e.mean; $n = 8$–10).

Effects of selective 5-HT and catecholamine uptake inhibitors on 8-OH-DPAT-induced hypothermia

In the mouse, the selective 5-HT uptake inhibitors citalopram, fluvoxamine, panuramine (Blurton *et al.*, 1984) and Wy 27587 (Fletcher *et al.*, 1988; Billingsley *et al.*, 1989), administered orally 60 min before 8-OH-DPAT, dose-dependently attenuated 8-OH-DPAT-induced hypothermia (Figure 3). The ED_{50} values (doses required to attenuate hypothermia by 50%) for citalopram, fluvoxamine, panuramine and Wy 27587 were 2.9 (2.3–3.6), 8.0 (5.5–11.6), 8.7 (5.0–15.2) and 1.7 (1.2–2.4) mg kg^{-1}, orally, respectively. Figures in parentheses are 95% confidence limits determined by linear regression analysis. The top doses of citalopram and fluvoxamine (30 mg kg^{-1}) completely abolished hypothermia, whereas panuramine induced a maximum attenuation of 62% ($P < 0.001$) at 15 mg kg^{-1}, and Wy 27587 a 63% attenuation ($P < 0.001$) at 2.0 mg kg^{-1}. The 5-HT uptake inhibitors did not modify body temperatures significantly in their own right. In marked contrast to the mouse data, the 5-HT uptake inhibitors had no significant effect on 8-OH-DPAT-induced hypothermia in the rat up to doses (mg kg^{-1}) of 50 (fluvoxamine), 100 (panuramine) or 10 (Wy 27587). The selective noradrenaline uptake inhibitor, desipramine (up to a dose of 30 mg kg^{-1}), had no significant effect on 8-OH-DPAT-induced hypothermia in either species. The selective dopamine uptake inhibitor, nomifensin, slightly (by 33%) and significantly ($P < 0.01$) attenuated hypothermia at the top dose of 30 mg kg^{-1}, orally in the rat (Table 1B). In contrast, in the mouse, nomifensin (3 mg kg^{-1}, p.o.) significantly ($P < 0.05$) enhanced (by 52%) 8-OH-DPAT-induced hypothermia (Table 1A).

Effects of fenfluramine and 5-HTP/carbidopa on 8-OH-DPAT-induced hypothermia

In the mouse, fenfluramine (20 mg kg^{-1} i.p., administered 20 min before 8-OH-DPAT) markedly attenuated 8-OH-DPAT-induced hypothermia by 76% ($P < 0.001$). In this species, carbidopa (25 mg kg^{-1}, i.p., 60 min before 5-HTP) plus 5-HTP (50 mg kg^{-1}, s.c., 30 min before 8-OH-DPAT) virtually abolished the hypothermia induced by 8-OH-DPAT (93% attenuation; $P < 0.001$). These data are displayed in Figure 4.

Carbidopa/5-HTP (25 mg kg^{-1}, i.p./12.5–50 mg kg^{-1}, s.c.) had no significant effect on 8-OH-DPAT-induced hypothermia in the rat whereas fenfluramine, at the top dose of 5 mg kg^{-1}, i.p., slightly attenuated (by 26%; $P < 0.05$) the hypothermia (see Table 2). This dose of fenfluramine alone, however, significantly ($P < 0.01$) reduced body temperature prior to 8-OH-DPAT administration (Table 2).

Effects of lesioning and 5-HT depletion on 8-OH-DPAT-induced hypothermia and syndrome

By day 5 after i.c.v. administration to mice, 5,7-DHT had produced a 61% decrease in brain 5-HT content, and a 56% decrease in 5-HIAA levels (Table 3A). This pretreatment totally abolished the hypothermic response to 8-OH-DPAT (0.1–1.0 mg kg^{-1}, s.c.; Figure 5a). PCPA administration to mice reduced brain 5-HT levels by 54% and 5-HIAA levels by 65% (Table 3A). This pretreatment attenuated 8-OH-DPAT-induced hypothermia; an effect which was significant ($P < 0.05$) against 0.3 mg kg^{-1} of 8-OH-DPAT (Figure 6a).

5,7-DHT lesioning in the rat reduced brain 5-HT levels by 70% and 5-HIAA levels by 83% (Table 3B). This pretreatment attenuated 8-OH-DPAT-induced hypothermia only at the top dose of 8-OH-DPAT (1.0 mg kg^{-1} by 52%; $P < 0.01$) and had no effect on the hypothermic responses to lower doses (Figure 5b). 5,7-DHT-lesioned rats were irritable, hyperreactive and aggressive, and had a slightly (non-significant) higher initial

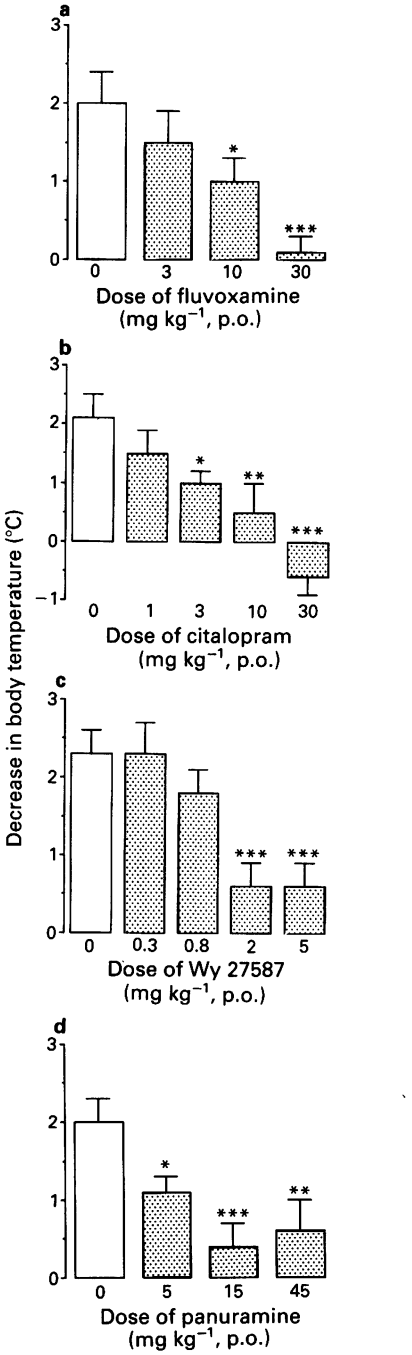

Figure 3 Effects of selective 5-hydroxytryptamine (5-HT) uptake inhibitors on 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT)-induced hypothermia in the mouse. (a) Fluvoxamine, (b) citalopram, (c) Wy 27587 and (d) panuramine were administered orally 60 min before the s.c. injection of 8-OH-DPAT (0.8 mg kg^{-1}) or saline. Body temperatures were measured immediately before each drug injection, and at 15 and 30 min post-injection of 8-OH-DPAT. Data shown are the mean maximum decreases in body temperature occurring in this latter period. Error bars denote ± s.e.mean ($n = 8$). Open columns show hypothermic response to 8-OH-DPAT in animals pretreated with vehicle. Stippled columns show hypothermic responses to 8-OH-DPAT in animals pretreated with 5-HT uptake inhibitors. The 5-HT uptake inhibitors alone and the s.c. injection of saline had no significant effect on body temperature. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; (one-way ANOVA followed by Student's unpaired t test cf. vehicle/8-OH-DPAT-treated group).

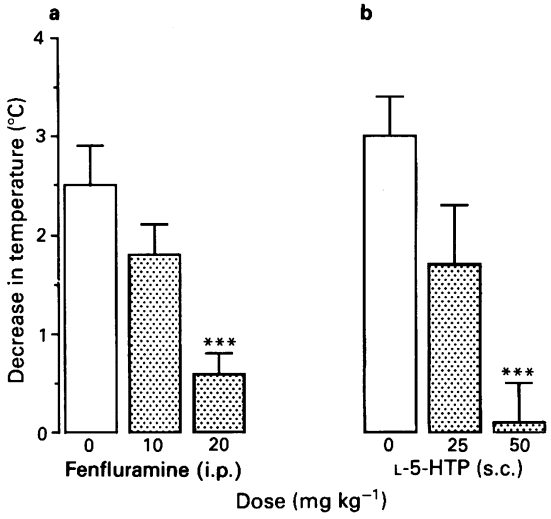

Figure 4 Effects of (a) fenfluramine and (b) carbidopa/L-5-hydroxytryptophan (L-5-HTP) on 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced hypothermia in the mouse. Fenfluramine (5–20 mg kg^{-1}) or vehicle were administered i.p. 20 min prior to the s.c. injection of 8-OH-DPAT (0.8 mg kg^{-1}) or saline. Carbidopa (25 mg kg^{-1}, i.p.) was administered 60 min before the s.c. injection of L-5-HTP (25–100 mg kg^{-1}). 8-OH-DPAT (0.8 mg kg^{-1}) or saline were injected s.c. a further 30 min after L-5-HTP. Body temperatures were recorded immediately before each drug injection, and at 15 and 30 min after injection of 8-OH-DPAT. Data shown are the mean maximum decreases in body temperature occurring after injection of 8-OH-DPAT. Error bars denote ± s.e.mean ($n = 8$). Open columns show hypothermic response to 8-OH-DPAT in animals pretreated with vehicle. Stippled columns show hypothermic responses to 8-OH-DPAT in animals pretreated with fenfluramine or L-5-HTP. *** $P < 0.001$ (one-way ANOVA followed by Student's unpaired t test cf. vehicle/8-OH-DPAT-treated group).

body temperature than controls.The 8-OH-DPAT behavioural syndrome was significantly ($P < 0.05$) enhanced in 5,7-DHT-lesioned rats (Figure 7). PCPA pretreatment in rats reduced brain levels of 5-HT and 5-HIAA by 90% and 80% respectively (Table 3B), and significantly ($P < 0.05$) enhanced the hypothermic responses to 0.125 and 0.5 mg kg^{-1} of 8-OH-DPAT (Figure 6b).

Discussion

The results of our experiments on 8-OH-DPAT-induced hypothermia in the mouse are clearly defined, in that both prior depletion of neuronal 5-HT and lesioning of presynaptic function (with PCPA or 5,7-DHT respectively) markedly attenuated the hypothermic response. These observations are consistent with previous work (Goodwin *et al.*, 1985) which indicated that the 5-HT$_{1A}$ receptors mediating 8-OH-DPAT-induced hypothermia in the mouse are located presynaptically on 5-HT neurones. Our finding that agents which facilitate 5-HT release acutely, also attenuated 8-OH-DPAT-induced hypothermia in the mouse, suggests that an autoreceptor-mediated inhibition of 5-HT release, on to an (as yet) unidentified postsynaptic 5-HT receptor, is primarily responsible for this hypothermic response. However, in the rat, the pharmacological profile of 8-OH-DPAT-induced hypothermia differs markedly from that in the mouse. In the rat, selective 5-HT uptake inhibitors had no significant effect, even at doses known to inhibit 5-HT re-uptake maximally in this species (our own unpublished data; see also Fletcher *et al.*, 1988). Similarly, the administration of 5-HTP, following peripheral decarboxylase inhibition, had no significant effect on the hypothermic response in rats, but virtually abolished the response to 8-OH-DPAT in the mouse. Also the 5-HT releasing agent, fenfluramine, markedly inhibited hypothermia in the mouse, but only slightly attenuated the response to 8-OH-DPAT in the rat, at a dose which itself reduced body temperature significantly prior to 8-OH-DPAT administration. It is feasible that this modest (26%) apparent attenu-

Table 1 Effect of the dopamine uptake inhibitor, nomifensin, on 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced hypothermia in (A) mouse and (B) rat

		Treatment 1 ($mg\,kg^{-1}$, p.o.)	*Treatment 2* ($mg\,kg^{-1}$, s.c.)	Δt (°C) (0–30 min post—8-OH-DPAT)	% *change*
A	*Mouse*	Vehicle	Saline	+0.2 ± 0.1	—
		Vehicle	8-OH-DPAT (0.8)	−2.0 ± 0.3	—
		Nomifensin (0.3)	8-OH-DPAT (0.8)	−1.9 ± 0.4	−5
		Nomifensin (1.0)	8-OH-DPAT (0.8)	−2.3 ± 0.3	+13
		Nomifensin (3.0)	8-OH-DPAT (0.8)	−3.1* ± 0.3	+52
		Nomifensin (10.0)	8-OH-DPAT (0.8)	−2.8* ± 0.2	+40
		Nomifensin (10.0)	Saline	−0.1 ± 0.1	—
B	*Rat*	Vehicle	Saline	+0.5 ± 0.1	—
		Vehicle	8-OH-DPAT (0.3)	−1.6 ± 0.2	—
		Nomifensin (3.0)	8-OH-DPAT (0.3)	−1.8 ± 0.2	+8
		Nomifensin (10.0)	8-OH-DPAT (0.3)	−1.4 ± 0.1	−8
		Nomifensin (30.0)	8-OH-DPAT (0.3)	−0.8** ± 0.1	−33
		Nomifensin (30.0)	Saline	+0.3 ± 0.1	—

Mice or rats ($n = 8$) were treated orally with nomifensin or vehicle 60 min prior to the s.c. injection of 8-OH-DPAT ($0.8\,mg\,kg^{-1}$ for mice; $0.3\,mg\,kg^{-1}$ for rats) or saline. Body temperatures were recorded immediately before each drug treatment, and at 15 and 30 min post-injection of 8-OH-DPAT or saline.

Δt shows the decrease in body temperature (mean ± s.e.mean) occurring in this latter period. Nomifensin alone had no significant effect on the body temperature of mice or rats prior to injection of 8-OH-DPAT or saline. Nomifensin potentiated 8-OH-DPAT-induced hypothermia in the mouse, but at a high dose attenuated it in the rat. * $P < 0.05$, ** $P < 0.01$ (one-way ANOVA followed by Student's unpaired t test cf. vehicle/8-OH-DPAT-treated groups).

Table 2 Effect of (A) fenfluramine, or (B) carbidopa/L-5-hydroxytryptophan (L-5-HTP) on 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced hypothermia in the rat

	Treatment 1 ($mg\,kg^{-1}$)	*Treatment 2* ($mg\,kg^{-1}$ s.c.)	Δt(°C) (0–30 min post—8-OH-DPAT)
A	Vehicle (i.p.)	Saline	+0.1 ± 0.1
	Vehicle (i.p.)	8-OH-DPAT (0.3)	−1.8 ± 0.2
	Fenfluramine (1.25 i.p.)	8-OH-DPAT (0.3)	−1.7 ± 0.1
	Fenfluramine (2.50 i.p.)	8-OH-DPAT (0.3)	−2.0 ± 0.2
	Fenfluramine (5.00 i.p.)	8-OH-DPAT (0.3)	−1.3 ± 0.1*
B	Vehicle (s.c.)	Saline	0.0 ± 0.1
	Vehicle (s.c.)	8-OH-DPAT (0.3)	−1.6 ± 0.3
	L-5-HTP (12.5 s.c.)	8-OH-DPAT (0.3)	−1.6 ± 0.3
	L-5-HTP (25.0 s.c.)	8-OH-DPAT (0.3)	−1.4 ± 0.2
	L-5-HTP (50.0 s.c.)	8.OH-DPAT (0.3)	−2.0 ± 0.3
	L-5-HTP (50.0 s.c.)	Saline	0.0 ± 0.1

(A) Fenfluramine ($1.25–5.0\,mg\,kg^{-1}$) or vehicle were administered i.p. to rats ($n = 8$) 20 min before the s.c. injection of 8-OH-DPAT ($0.3\,mg\,kg^{-1}$) or saline. Administration of fenfluramine alone ($5.0\,mg\,kg^{-1}$, i.p.) significantly ($P < 0.01$) reduced body temperature prior to 8-OH-DPAT administration (i.e. changes in body temperatures 20 min following treatment 1 were: vehicle + 0.2 ± 0.1; fenfluramine (5) −0.8 ± 0.1).

(B) Carbidopa ($25\,mg\,kg^{-1}$, i.p.) was administered to rats 60 min before the s.c. injection of L-5-HTP ($12.5–50\,mg\,kg^{-1}$) or saline. 8-OH-DPAT ($0.3\,mg\,kg^{-1}$) or saline were injected s.c. a further 30 min later. Body temperatures were recorded immediately before each drug treatment, and at 15 and 30 min post-injection of 8-OH-DPAT or saline. Δt shows the change in body temperature (mean ± s.e.mean; $n = 8$) occurring in this latter period.

* $P < 0.05$ (one-way ANOVA followed by Student's unpaired t test cf. vehicle/8-OH-DPAT treated group).

Table 3 Effects of pretreatment with 5,7-dihydroxytryptamine (5,7-DHT) or *p*-chlorophenylalanine (PCPA) on 5-hydroxytryptamine (5-HT) and 5-hydroxyindolacetic acid (5-HIAA) levels in (A) the mouse and (B) the rat

			Brain monoamine and metabolite levels ($ng\,g^{-1}$ wet weight)			
		Treatments	*5-HT*	*5-HIAA*	*NA*	*Dopamine*
A	Mouse	Saline (5 µl/mouse i.c.v.)	506 ± 15	259 ± 13	290 ± 27	715 ± 45
		5,7-DHT (50 µg/mouse i.c.v.)	199 ± 23*** (−61%)	114 ± 14*** (−56%)	255 ± 42 (−16%)	733 ± 39 (+3%)
		Vehicle ($10\,ml\,kg^{-1}$ p.o. ×2)	401 ± 24	239 ± 20	249 ± 10	797 ± 25
		PCPA ($750\,mg\,kg^{-1}$ p.o. ×2)	191 ± 36*** (−54%)	85 ± 25*** (−65%)	239 ± 10 (−4%)	713 ± 35 (−11%)
B	Rat	Saline (2.5 µl/rat i.t.v.)	491 ± 25	224 ± 11	392 ± 16	956 ± 21
		5,7-DHT (150 µg/rat i.t.v.)	125 ± 19*** (−75%)	49 ± 8*** (−78%)	275 ± 16** (−30%)	954 ± 17 (0%)
		Vehicle ($2.5\,ml\,kg^{-1}$ p.o. ×2)	407 ± 15	241 ± 7	366 ± 15	769 ± 18
		PCPA ($200\,mg\,kg^{-1}$ p.o. ×2)	36 ± 4*** (−91%)	20 ± 1*** (−92%)	247 ± 10 (−5%)	730 ± 26 (−5%)

Mice were injected i.c.v. with ascorbate/saline (5 µl) or 5,7-DHT (50 µg base) 5 days before determination of whole brain (minus cerebellum) monoamine levels. Rats were injected i.t.v. (intra-third ventricle) with ascorbate/saline (2.5 µl) or 5,7-DHT (150 µg base) 10 days before determination of brain monoamine levels. PCPA or vehicle were administered p.o. to both mice and rats on days 1 and 2, with brain monoamine levels determined by h.p.l.c. with electrochemical detection on day 5. Values are mean ± s.e.mean levels from 6–8 animals. ** $P < 0.01$; *** $P < 0.001$ (Student's unpaired t test cf. saline or vehicle-pretreated control groups).

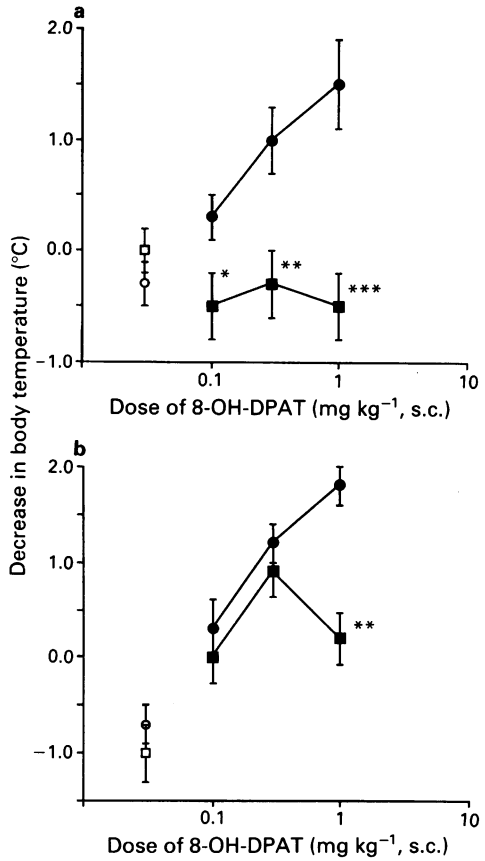

Figure 5 Effects of 5,7-dihydroxytryptamine (5,7-DHT) lesions on 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT)-induced hypothermia in (a) the mouse and (b) the rat. Mice were injected i.c.v. with 5 μl of ascorbate/saline (●) or 50 μg base of 5,7-DHT (■) 5 days before the s.c. administration of 8-OH-DPAT (0.1–1.0 mg kg^{-1}) or saline. Rats were injected into the third ventricle (i.t.v.) with 2.5 μl of ascorbate/saline (●) or 150 μg base of 5,7-DHT (■) 10 days before the s.c. administration of 8-OH-DPAT or saline. Body temperatures were measured immediately before, and at 15 and 30 min after 8-OH-DPAT. The hypothermic response to 8-OH-DPAT was measured as the maximum decrease in body temperature occurring in this latter period. Open symbols show changes in body temperature after s.c. injection of saline in animals pretreated with ascorbate/saline (○) or 5.7-DHT (□). Data points are mean ± s.e.mean (n = 8). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, (ANOVA followed by Student's unpaired t test cf. ascorbate/saline pretreated groups).

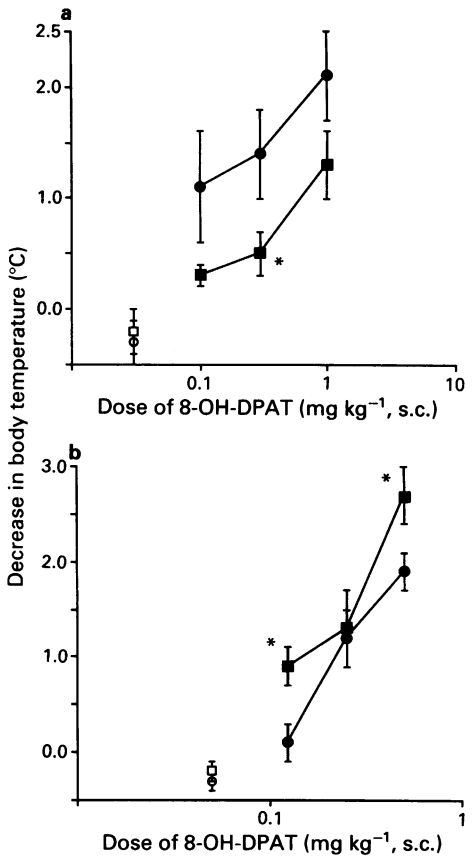

Figure 6 Effect of p-chlorophenylalanine (PCPA) pretreatment on 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced hypothermia in (a) the mouse and (b) the rat. PCPA (■) (750 mg kg^{-1} for mice; 200 mg kg^{-1} for rats) or vehicle (●) were administered orally on days 1 and 2. On day 5 groups of 8 animals were dosed s.c. with saline or 8-OH-DPAT (0.1–1.0 mg kg^{-1} for mice; 0.125–0.5 mg kg^{-1} for rats) or saline. Body temperatures were measured immediately before, and at 15 and 30 min post-injection of 8-OH-DPAT. The hypothermic response to 8-OH-DPAT was measured as the maximum decrease in body temperature occurring in this latter period. Open symbols show changes in body temperature after s.c. injection of saline in animals pretreated with vehicle (○) or PCPA (□). Data points are mean ± s.e.mean (n = 8). * $P < 0.05$ (ANOVA followed by Student's unpaired t test).

ation in the rat could result from stimulation of postsynaptic 5-HT$_{1A}$ receptors following fenfluramine-induced 5-HT release, which alone might induce a slight hypothermia prior to administration of 8-OH-DPAT. Fenfluramine alone, had no significant effects on body temperature in the mouse. The present experiments were performed at a near normal ambient temperature of 20°C. In rats housed at ambient temperatures of 28°C or greater, fenfluramine elicits hyperthermia, an effect thought to be mediated via 5-HT release onto postsynaptic 5-HT$_2$ receptors (Blackburn *et al.*, 1985; 1990). However, previous work also indicates that fenfluramine has little effect on or actually decreases the body temperature of rats maintained at ambient temperatures of 20 or 23°C (Sulpizo *et al.*, 1978; Preston *et al.*, 1990). The different dose-ranges of fenfluramine used for rats or mice in the present study reflect the fact that these two species appear to differ markedly in their sensitivity to the 5-HT-releasing action of this drug. For example, we, and many other workers, find that fenfluramine exerts a marked anorectic action in the rat over the dose-range employed in this study (Blundell & Latham, 1978; Garattini *et al.*, 1978), whereas higher doses are required to inhibit food consumption in the mouse (our unpublished data).

A 90% depletion of brain 5-HT levels in the rat, by prior administration of PCPA, did not attenuate 8-OH-DPAT-induced hypothermia in the rat. On the contrary, PCPA pretreatment significantly increased the hypothermic response to the lowest and highest doses of 8-OH-DPAT, although the response to the intermediate dose was unchanged. Lesioning of 5-HT neurones with 5,7-DHT had no effect on the hypothermic responses to the lower doses of 8-OH-DPAT, but significantly attenuated the response to the top dose (1.0 mg kg^{-1}). However, it should be noted that 5,7-DHT pretreatment also significantly increased the behavioural stimulation (5-HT syndrome) induced by 8-OH-DPAT at a dose of 0.3 mg kg^{-1} (Figure 7). Therefore, enhanced motor responses to higher doses of 8-OH-DPAT in lesioned animals may raise body temperature non-specifically, thereby attenuating hypothermia. The stereotypy induced by the top dose of 8-OH-DPAT was not increased significantly in lesioned animals. This may reflect the fact that the behavioural syndrome response was near maximal in sham-operated control rats at this dose. 8-OH-DPAT-induced motor stimulation *per se* was not measured in these experiments, but may have been enhanced in lesioned animals. This point should be investigated in subsequent experiments.

A possible explanation for the discrepancy between our data and those of Goodwin *et al.* (1987) could be related to

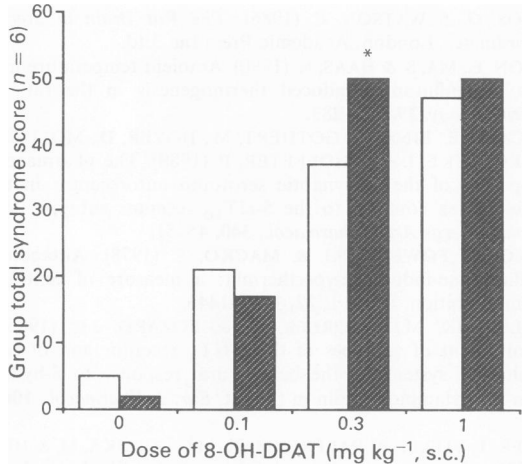

Figure 7 Effects of 5,7-dihydroxytryptamine (5,7-DHT) lesioning on the 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced behavioural syndrome in the rat. Rats were sham-treated with ascorbate/saline (2.5 μl) (open columns) or treated with 5,7-DHT (150 μg base) (hatched columns) injected into the 3rd ventricle (i.t.v.) by stereotaxic techniques. On day 10 post-lesioning, animals were injected s.c. with saline or 8-OH-DPAT (0.1–1.0 mg kg^{-1}); 10 min later the severity of the behavioural syndrome induced by 8-OH-DPAT was assessed on a scale 0–10 for each rat. Syndrome scores for each treatment group ($n = 6$) were summed (max. possible score = 60). * $P < 0.05$ (Mann-Whitney U-test cf. sham-operated animals).

the fact that, as these authors reported, the dose-response curve for 8-OH-DPAT-induced hypothermia in the rat is biphasic. This appears to be a result of the general motor stimulation and behavioural syndrome which occurs at higher doses of 8-OH-DPAT. Since Goodwin *et al.* (1987) used a high challenge dose of 8-OH-DPAT (0.75 mg kg^{-1}, s.c.) relative to the dose inducing a maximum hypothermic response (0.25 mg kg^{-1}), it is feasible that an enhanced postsynaptic receptor sensitivity induced by 5-HT depletion resulted in a more severe syndrome, thereby shifting the hypothermic response further along the down-slope of the dose-response curve. An apparent attenuation of 8-OH-DPAT-induced hypothermia would be observed under these circumstances.

The selective noradrenaline uptake inhibitor, desipramine, had no effect on 8-OH-DPAT-induced hypothermia in either species, suggesting that the release of noradrenaline is not involved in this response. However, the selective dopamine reuptake inhibitor, nomifensin, significantly enhanced the hypothermic response to 8-OH-DPAT in the mouse, possibly suggesting that dopamine release is involved secondarily in this response. Such an involvement is also consistent with the original observation of Goodwin *et al.* (1985), that the dopamine antagonist, haloperidol, attenuated 8-OH-DPAT-induced hypothermia in the mouse. We have found that other dopamine antagonists (e.g. fluphenazine and sulpiride) also inhibit 8-OH-DPAT-induced hypothermia in the mouse (unpublished observations). However, the lack of activity of flupenthixol (Goodwin *et al.*, 1985; and our own unpublished observations) in this model is an anomalous observation which is not consistent with an involvement of dopamine release in the expression of 8-OH-DPAT-induced hypothermia. In the rat, haloperidol did not significantly modify the hypothermic response to 8-OH-DPAT (unpublished observation), whereas nomifensin attenuated it slightly at high doses. This latter effect may have been due to a motor stimulant action of nomifensin, inducing a slight rise in body temperature which non-specifically attenuated the hypothermia. This suggestion is supported by the results of our own experiments demonstrating significant increases in open field activity of rats at these doses of nomifensin (unpublished data). It appears, therefore, that the mechanism underlying 8-OH-DPAT-induced hypothermia in the mouse and the rat, may also differ in terms of dopamine involvement.

In conclusion, our data indicate a fundamental difference in the mechanisms by which 8-OH-DPAT induces hypothermia in the mouse and rat. All data are consistent with a presynaptic 5-HT$_{1A}$ receptor agonist action in the mouse, leading to an attenuation of 5-HT release whereas the 5-HT$_{1A}$ receptor mediating hypothermia in the rat appears to be located postsynaptically with respect to 5-HT neurones.

Other differences are also apparent, in that there is no clear evidence for an involvement of dopamine release in the rat, whereas, in the mouse, opposing effects of a dopamine uptake inhibitor and several dopamine D_2 receptor antagonists, may indicate an involvement of dopamine in the mechanism of 8-OH-DPAT-induced hypothermia in this species.

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Determination of the role of noradrenergic and 5-hydroxytryptaminergic neurones in postsynaptic α_2-adrenoceptor desensitization by desipramine and ECS

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1 Experiments were conducted to determine the respective roles which noradrenergic and 5-hydroxytryptaminergic neurones play in the down-regulation of postsynaptic α_2-adrenoceptors by desipramine and electroconvulsive shock (ECS). The functional status of these receptors was monitored by use of clonidine-induced mydriasis in conscious mice.

2 Mydriasis to clonidine (0.1 mg kg^{-1}, i.p.) was markedly attenuated by administration of either desipramine (10 mg kg^{-1}, i.p.) for 14 days or ECS (200 V, 2 s) given five times over ten days confirming our previous observations.

3 The neurotoxin, DSP-4 (100 mg kg^{-1}, i.p. × 2), reduced brain noradrenaline levels by 64% and abolished the mydriasis induced by the noradrenaline releasing agent and reuptake inhibitor, methamphetamine, without significantly altering the response to clonidine, confirming our earlier results. This lesion prevented the attenuation of clonidine mydriasis by repeated administration of desipramine, but not ECS.

4 Lesioning of central 5-hydroxytryptaminergic neurones with 5,7-dihydroxytryptamine (75 μg, i.c.v.) had no influence on the reduction in clonidine mydriasis produced by repeated administration of either desipramine or ECS.

5 Since noradrenergic neurones are essential for the desensitization of postsynaptic α_2-adrenoceptors by desipramine, it indicates that this effect is probably the result of increased synaptic noradrenaline levels. This mechanism is not responsible for the change induced by ECS because this adaptation is independent of an intact noradrenergic input. 5-HT-containing neurones do not play a permissive role in the down-regulation of postsynaptic α_2-adrenoceptors by either antidepressant treatment.

Keywords: Clonidine-induced mydriasis; α_2-adrenoceptors; postsynaptic α_2-adrenoceptors; brain; antidepressants; antidepressant effects; desipramine; electroconvulsive shock

Introduction

It is now accepted that prolonged administration of many antidepressant drugs produces changes in the function of the monoamine neurotransmitters, noradrenaline and 5-hydroxytryptamine (5-HT). The most familiar of these adaptive effects are probably the down-regulation of β-adrenoceptors (Schultz, 1976; Vetulani *et al.*, 1976a,b) and 5-HT$_2$ receptors (Peroutka & Snyder, 1980; Blackshear *et al.*, 1982; Goodwin *et al.*, 1984; Metz & Heal, 1986). However, the concept that down-regulation results exclusively from an increase in the synaptic concentrations of these neurotransmitters is too simplistic to explain the actions of all antidepressant treatments. For example, the down-regulation of β-adrenoceptors by desipramine is dependent upon intact noradrenergic function (Vetulani *et al.*, 1976a; Dooley *et al.*, 1983; Hall *et al.*, 1984), whereas electroconvulsive shock (ECS) decreases these receptors even after destruction of noradrenergic neurones (Vetulani & Sulser, 1975; Kellar *et al.*, 1981; Dooley *et al.*, 1987). In addition, there is also evidence to show that antidepressant-induced adrenoceptor desensitization involves a functional interplay between central noradrenergic and 5-hydroxytryptaminergic neurones. Thus, inhibition of 5-HT synthesis or denervation with 5,7-dihydroxytryptamine (5,7-DHT) prevents either the down-regulation of β-adrenoceptors by antidepressants (Brunello *et al.*, 1982; 1985; Nimgaonkar *et al.*, 1985) or, possibly, the maintenance of this effect (Asakura *et al.*, 1987).

Recently, we have demonstrated that clonidine-induced mydriasis measured in conscious C57/B1/6 mice is specifically mediated by postsynaptic α_2-adrenoceptors in the brain (Heal *et al.*, 1989a,b). Moreover, this functional response is attenuated after repeated administration of either antidepressants which increase central noradrenergic function or repeated ECS (Heal *et al.*, 1991). In experiments analogous to those previously conducted on β-adrenoceptors, we have now determined the roles which noradrenergic and 5-hydroxytryptaminergic neurones play in the attenuation of postsynaptic α_2-adrenoceptor function by administration of desipramine and ECS.

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Methods

Animals

Adult male C57/B1/601a mice (Olac) weighing 25–30 g were used. They were housed in groups of six to ten on a 12 h light/dark cycle (commencing 06 h 00 min) at a temperature of 21°C and 55% humidity. Mice were allowed free access to food and water.

Lesioning of noradrenaline- and 5-HT-containing neurones in the brain

Destruction of brain noradrenergic neurones was carried out by use of the selective neurotoxin, DSP-4. Mice were pretreated with zimeldine (5 mg kg^{-1}, i.p.) to protect central 5-HT-containing neurones and DSP-4 (100 mg kg^{-1}, i.p.) was administered 30 min later. This procedure was repeated seven days later. After a further three days, verification of extensive noradrenergic denervation was initially obtained by measurement of methamphetamine (0.5 mg kg^{-1}, i.p.)-induced

mydriasis in all DSP-4 lesioned mice as this response is abolished in mice after depletion of brain noradrenaline levels by approximately 75% (Heal et al., 1989b). To demonstrate that no shift in the basal mydriasis response had occurred after DSP-4 lesioning, groups of mice were randomly selected from the methamphetamine non-responders and sham-lesioned controls and their response to clonidine (0.1 mg kg^{-1}, i.p.) was determined.

5-Hydroxytryptaminergic neurones were lesioned by use of the selective neurotoxin, 5,7-DHT. Mice were initially pretreated with desipramine (25 mg kg^{-1}, i.p.) to protect noradrenaline-containing neurones and 15 min later the animals were anaesthetized with hexobarbitone (50 mg kg^{-1}, i.p.). 5,7-DHT (75 μg) dissolved in 4 μl ice-cold saline containing 0.4 mg ml^{-1} ascorbic acid was injected intracerebroventricularly (i.c.v.) with the stereotaxic apparatus described by Heal (1984). Sham-lesioned mice were injected with vehicle (4 μl, i.c.v.). Mice were then housed individually and left for 14 days to recover. Verification of substantial 5-HT depletion was initially performed by measurement of 5-methoxy-N,N-dimethyltryptamine-induced head-twitches and finally by determination of neurotransmitter concentrations by high performance liquid chromatography (h.p.l.c.) with electrochemical detection. Full details of these procedures, together with the reduction in 5-HT levels, are given in Heal et al. (1990). To demonstrate that destruction of 5-HT-containing neurones had not affected α_2-adrenoceptor-mediated mydriasis, groups of mice were randomly selected from the acceptable 5,7-DHT-lesioned and sham-lesioned animals and these were tested with clonidine (0.1 mg kg^{-1}, i.p.).

Administration of desipramine and electroconvulsive shock

Mice injected with DSP-4 (100 mg kg^{-1}, i.p. × 2) or saline were each divided into two subgroups. They were injected with desipramine (10 mg kg^{-1}, i.p.) or saline (0.25 ml, i.p.) once daily for 14 days. In a second experiment, subgroups of DSP-4-treated and sham-lesioned mice were anaesthetized with a constant flow air-halothane mixture and given an ECS (200 V, 2 s) five times over 10 days (Mon, Wed, Fri, Mon, Wed). Controls were anaesthetized.

Groups of mice injected with 5,7-DHT (75 μg, i.c.v.) or saline-ascorbate (4 μl, i.c.v.) were subdivided and treated with desipramine or ECS according to the protocols described above.

Clonidine-induced mydriasis was measured 24 h after the final desipramine or ECS treatment.

Measurement of α_2-adrenoceptor-mediated mydriasis

A Wild M1 binocular microscope with graticule scale in one eyepiece was used to measure pupil diameter. Illumination of the microscope was provided by a Swift light box with the voltage set at 6 V (light intensity 2500 lux). The procedure was carried out in an artificially lit room (light intensity 650 lux). The mouse was held beneath the microscope and its pupil diameter was read off in eyepiece units. This figure was later converted to millimetres. The mouse was injected with either clonidine (0.1 mg kg^{-1}, i.p.) or methamphetamine (0.5 mg kg^{-1}, i.p.) and its pupil diameter was measured again 10 min later. A dose of 0.1 mg kg^{-1} of clonidine was chosen because it allowed for the detection of either enhancement or inhibition of this response (Heal et al., 1989a).

Measurement of brain monoamine concentrations by h.p.l.c. with electrochemical detection

Measurement of brain monoamines (noradrenaline, 5-HT and dopamine) was performed by h.p.l.c. with electrochemical detection to confirm the selectivity and extent of the DSP-4 and 5,7-DHT lesioning procedures. All lesioned and sham-lesioned mice used in the desipramine and ECS experiments were killed 24 h after the measurement of clonidine-induced mydriasis. Brains were homogenized in five volumes (w/v) of 0.1 M perchloric acid containing 0.4 mM sodium metabisulphite (antioxidant) and 0.8 μM isoprenaline (internal standard) in a Polytron PT 10–35 disruptor (setting 5–6). After centrifugation at 1100 g for 15 min at 4°C and 15000 g for 5 min at 4°C, 30 μl of the resulting supernatant was injected onto the h.p.l.c. system for the determination of monoamine concentrations. This comprised a DuPont 870 pump (flow rate 1 ml min^{-1}) connected via a WISP 712 refrigerated autoinjector to a 5 μm Hypersil ODS 1 reversed-phase analytical column (length 250 × 4.6 mm i.d.) maintained at 45°C and protected by a Brownlee Aquapore RP-300 precolumn (length 30 × 4.6 mm i.d.). The h.p.l.c. mobile phase was 0.1 M sodium dihydrogen orthophosphate-orthophosphoric acid buffer pH 3.2 containing 16% v/v methanol, 2.8 mM 1-octane sulphonic acid sodium salt and 0.7 mM EDTA. Noradrenaline, 5-HT and dopamine were detected by use of a BAS LC-4A amperometric detector with a TL-5 flow-cell set at a potential of +0.75 V versus an Ag/AgCl reference electrode.

Drugs and reagents

Drugs were obtained from the following sources: clonidine HCl, desipramine HCl, 5,7-dihydroxytryptamine creatinine sulphate (5,7-DHT), hexobarbitone, methamphetamine HCl (Sigma, Poole); (−)-ascorbic acid (FSA, Loughborough); halothane (May and Baker, Dagenham); N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4; Semat, St. Albans). Drugs for intraperitoneal injection were dissolved in 0.9% (w/v) sodium chloride solution (saline) and were administered in weight-related doses (0.1 ml 10 g^{-1} body weight).

Reagents for h.p.l.c. analysis were of the highest purity available and were obtained from the following sources: dopamine HCl, 5-hydroxytryptamine creatinine sulphate (5-HT), isoprenaline HCl, noradrenaline HCl, sodium metabisulphite (Sigma, Poole); perchloric acid, orthophosphoric acid (BDH, Poole); 1-octane sulphonic acid sodium salt, sodium dihydrogen orthophosphate (FSA, Loughborough); ethylenediaminetetraacetic acid disodium salt (EDTA) (Aldrich, Gillingham); methanol (Rathburn Chemicals, Walkerburn). All water was distilled and deionised before used.

Statistics

Results were statistically evaluated by Student's unpaired t test.

Results

Effects of noradrenergic denervation

Effects of DSP-4 lesioning on methamphetamine- and clonidine-induced mydriasis DSP-4 (100 mg kg^{-1}, i.p. × 2) was administered to mice as outlined in Methods. Mice were tested for their mydriatic response to methamphetamine (0.5 mg kg^{-1}, i.p.) three days after the second DSP-4 treatment. DSP-4 lesioning abolished the methamphetamine-induced response when compared with sham-lesioned controls (Figure 1a). A subgroup of these mice was tested with clonidine (0.1 mg kg^{-1}, i.p.) 24 h later. Although DSP-4 lesioning produced a small enhancement of this response, the potentiation was not significant (Figure 1b).

Influence of DSP-4 lesioning on the attenuation of clonidine-induced mydriasis by desipramine or ECS Groups of mice injected with DSP-4 (100 mg kg^{-1}, i.p. × 2) or saline were injected with desipramine (10 mg kg^{-1}, i.p.) once daily for 14 days, while controls received saline (0.25 ml, i.p.). When the

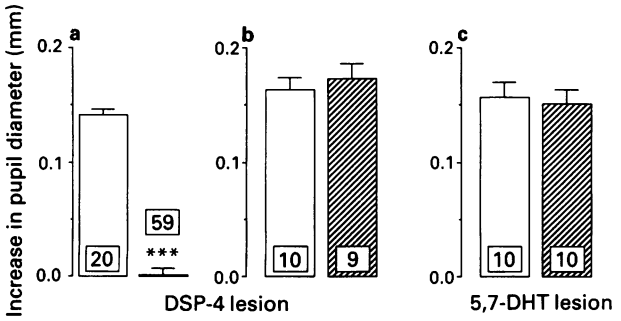

Figure 1 The effects of DSP-4 and 5,7-dihydroxytryptamine (5,7-DHT) lesioning on α_2-adrenoceptor-mediated mydriasis responses. (a and b) Groups of mice were injected with DSP-4 (100 mg kg^{-1}, i.p. × 2; hatched columns) or saline (0.25 ml, i.p. × 2; open columns). (c) Groups of mice were injected with 5,7-DHT (75 μg, i.c.v; hatched columns) or saline (4 μl, i.c.v.; open columns). All lesioning procedures are fully described in Methods. The results are the increase in pupil diameter (mm) (s.e.mean shown by vertical bars) measured 10 min after (a) methamphetamine (0.5 mg kg^{-1}, i.p.) or (b and c) clonidine (0.1 mg kg^{-1}, i.p.). The number of mice tested is shown in each column. Significantly different from appropriate control group: *** $P < 0.001$.

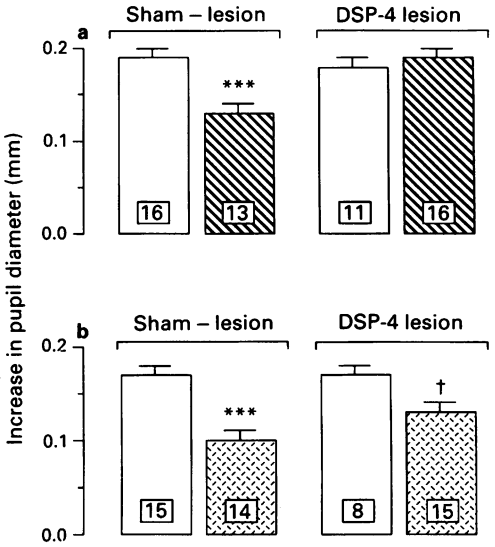

Figure 2 The influence of noradrenergic denervation on the attenuation of clonidine-induced mydriasis by desipramine or electroconvulsive shock (ECS). Groups of DSP-4 (100 mg kg^{-1}, i.p. × 2)-treated and sham-lesioned mice were: (a) injected with desipramine (10 mg kg^{-1}, i.p.; hatched columns) or saline (0.25 ml, i.p.; open columns) once daily for 14 days or (b) given an ECS (200 V, 2 s; stippled columns) or halothane (open columns) five times over 10 days. Twenty-four hours after the final treatment, the mydriasis induced by clonidine (0.1 mg kg^{-1}, i.p.) was measured. The results are increase in pupil diameter (mm) (s.e.mean shown by vertical bars) measured 10 min after clonidine injection with the number of mice tested shown in each column. Significantly different from appropriate control group: † $P < 0.02$; *** $P < 0.001$.

mydriasis response to clonidine (0.1 mg kg^{-1}, i.p.) was measured 24 h after the final dose of desipramine, DSP-4 lesioning was found to prevent the attenuation of clonidine-induced mydriasis by repeated desipramine treatment (Figure 2a).

When groups of DSP-4-treated and sham-lesioned mice were given either an ECS (200 V, 2 s) five times over 10 days or halothane, DSP-4 treatment did not influence the reduction in clonidine-induced mydriasis induced by repeated ECS (Figure 2b).

Effects of DSP-4 treatment on brain monoamine concentrations Mice were killed 24 h after the determination of clonidine-induced mydriasis and brain concentrations of noradrenaline, 5-HT and dopamine were measured by h.p.l.c. with electrochemical detection as detailed in Methods. DSP-4 (100 mg kg^{-1}, i.p. × 2) treatment markedly reduced brain noradrenaline concentrations with only small alterations in 5-HT and dopamine levels (Table 1). The extent of noradrenaline depletion produced by DSP-4 was almost identical in both the desipramine and ECS experiments (Table 1).

Effects of 5-hydroxytryptaminergic denervation

Effect of 5,7-DHT lesioning on clonidine-induced mydriasis Fifteen days after 5,7-DHT (75 μg, i.c.v.) injection, a subgroup of the mice was tested with clonidine (0.1 mg kg^{-1}, i.p.). 5,7-DHT lesioning (for extent of 5-HT lesion see Heal *et al.*, 1990) had no significant effect on the mydriasis response when compared with sham-lesioned controls (Figure 1c).

Influence of 5,7-DHT lesioning on the attenuation of clonidine-induced mydriasis by desipramine or ECS Groups of 5,7-DHT (75 μg, i.c.v.)-treated and sham-lesioned mice were injected with desipramine (10 mg kg^{-1}, i.p.) once daily for 14 days, while controls received saline (0.25 ml, i.p.). The attenuation of clonidine (0.1 mg kg^{-1}, i.p.)-induced mydriasis by repeated desipramine treatment was unaffected by 5,7-DHT lesioning (extent of lesioning given in Heal *et al.*, 1990), when measured 24 h after the final dose of this antidepressant (Figure 3a).

When groups of 5,7-DHT-lesioned and sham-lesioned mice were given either an ECS (200 V, 2 s) five times over 10 days or halothane, 5,7-DHT treatment (extent of lesioning given in Heal *et al.*, 1990) did not affect the attenuation of clonidine-induced mydriasis by repeated ECS (Figure 3b).

Table 1 The effects of DSP-4 lesioning on mouse brain monoamine concentrations

	Brain concentrations		
	Noradrenaline	*5-HT*	*Dopamine*
Desipramine experiment			
Sham-lesioned	707 ± 6 (29)	842 ± 8	1374 ± 9
DSP-4 (100 mg kg^{-1} × 2)	258 ± 3 (27)***	903 ± 11***	1284 ± 14***
% depletion	64	−7	7
ECS experiment			
Sham-lesioned	690 ± 6 (29)	792 ± 12	1503 ± 22
DSP-4 (100 mg kg^{-1} × 2)	235 ± 3 (23)***	754 ± 18*	1239 ± 15***
% depletion	66	5	18

Mice were lesioned with DSP-4 (100 mg kg^{-1}, i.p. × 2) as detailed in Methods. The results are brain monoamine concentration (ng g^{-1} tissue wet wt) ± s.e.mean. The numbers of mice used are shown in parentheses in the left hand column. Significantly different from appropriate sham-lesioned control group: * $P < 0.05$; *** $P < 0.001$.

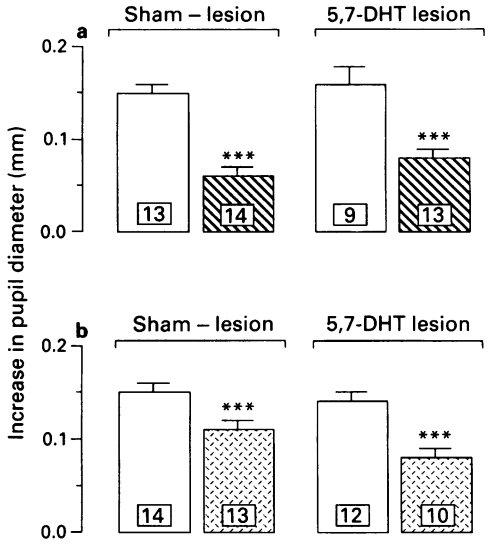

Figure 3 The influence of 5-hydroxytryptaminergic denervation on the attenuation of clonidine-induced mydriasis by desipramine or electroconvulsive shock (ECS). Groups of 5,7-dihydroxytryptamine (5,7-DHT, 75 μg, i.c.v.)-treated and sham-lesioned mice were: (a) injected with desipramine (10 mg kg^{-1}, i.p.; hatched columns) or saline (0.25 ml, i.p.; open columns) once daily for 14 days or (b) given an ECS (200 V, 2 s; stippled columns) or halothane (open columns) five times over 10 days. Twenty-four hours after the final treatment, the mydriasis induced by clonidine (0.1 mg kg^{-1}, i.p.) was measured. The results are increase in pupil diameter (mm) (with s.e.mean shown by vertical bars) measured 10 min after clonidine injection with the number of mice tested shown in each column. Significantly different from appropriate control group: *** $P < 0.001$.

Discussion

In this study, repeated administration of desipramine or ECS attenuated postsynaptic α_2-adrenoceptor function as indicated by clonidine-induced mydriasis and both findings are in agreement with earlier observations obtained by use of the mydriasis response in mice (Heal *et al.*, 1991) or rats (Menargues *et al.*, 1990). Since ligand-receptor binding techniques also predominantly monitor changes in the postsynaptic α_2-adrenoceptor population (U'Prichard *et al.*, 1980; Dooley *et al.*, 1983; Payvandi *et al.*, 1990), there is some value in comparing findings from these studies with those obtained with the clonidine-induced mydriasis model, although it should be noted that the anatomical sites involved are different, i.e. mydriasis results from activation of α_2-adrenoceptors in the Edinger-Westphal nucleus (Sharpe & Pickworth, 1981), whereas ligand-receptor binding is generally measured in the cortex. In agreement with the current findings, cortical α_2-adrenoceptors have been reported to be decreased by repeated treatment with desipramine or ECS (Smith *et al.*, 1981; Vetulani, 1982; Stanford & Nutt, 1982; Pilc & Vetulani, 1982; Heal *et al.*, 1987; 1989c). However, the former observation has been disputed by others (Johnson *et al.*, 1980; Asakura *et al.*, 1982; Sugrue, 1983; Stanford *et al.*, 1983).

DSP-4 lesioning was sufficiently complete to abolish the mydriasis evoked by methamphetamine, a noradrenaline releasing agent and reuptake inhibitor (Glowinski & Axelrod, 1965; Raiteri *et al.*, 1975), but did not suppress the response to the agonist, clonidine. However, in confirmation of our earlier findings (Heal *et al.*, 1989b), we observed that although there was a slight enhancement of clonidine-induced mydriasis, there was no evidence of marked denervation supersensitivity. Dooley *et al.* (1983) have also shown that DSP-4 lesioning did not increase α_2-adrenoceptors in all brain regions providing further evidence to indicate that certain populations of this receptor probably do not become supersensitive after denervation. If this hypothesis is correct, it provides the interesting phenomenon of a receptor that is easily down-regulated by increased noradrenergic function (Heal *et al.*, 1991) but is unresponsive to decreases in this neurotransmitter. Noradrenergic denervation prevented the decrease in clonidine-induced mydriasis produced by desipramine treatment, but did not impede the attenuation produced by ECS. These findings argue that the noradrenaline reuptake inhibitor, desipramine, probably causes adaption by increasing the synaptic concentrations of this neurotransmitter. By contrast, ECS almost certainly produces this effect by an indirect, but as yet, undefined mechanism and this hypothesis is supported by earlier studies showing that ECS has little effect on noradrenaline turnover or release (Nimgaonkar *et al.*, 1986; Green *et al.*, 1987). This finding of a differential necessity for an intact noradrenergic input in the attenuation of postsynaptic α_2-adrenoceptors by desipramine and ECS mirrors the previously reported requirements for the down-regulation of β-adrenoceptors by these two antidepressant treatments, i.e. noradrenergic neurones are essential for the down-regulation by desipramine, but not ECS (Vetulani & Sulser, 1975; Vetulani *et al.*, 1976a; Kellar *et al.*, 1981; Dooley *et al.*, 1983; 1987; Hall *et al.*, 1984). An alternative explanation is that it is the noradrenergic neurone (or possibly a cotransmitter contained within it), rather than noradrenaline *per se* which is essential for the down-regulation of α_2-adrenoceptors by desipramine. As an analogy, it has been reported that although 5-HT depletion with *p*-chlorophenylalanine does not influence desipramine-induced desensitization of β- or presynaptic α_2-adrenoceptors, 5,7-DHT lesioning abolished these effects, (Nimgaonkar *et al.*, 1986; Heal *et al.*, 1990) indicating that the 5-HT-containing neurone, but not 5-HT itself, is essential for the down-regulation process. However, this hypothesis is less plausible because studies which have examined the effects on mydriasis of monoamine depletion, as opposed to noradrenergic neuronal destruction, have clearly indicated that the latter do not have a functional role to play in the control of this response (Koss, 1979; Koss *et al.*, 1984).

In experiments to determine the influence which 5-HT function exerts on the antidepressant-induced attenuation of this postsynaptic α_2-adrenoceptor-mediated response, the indolamine-containing neurones were destroyed by intracerebroventricular injection of 5,7-DHT. By employing the method to determine sequentially clonidine-induced mydriasis and hypoactivity in the same animal (Heal *et al.*, 1989b), the mice used in the present study were those previously used to determine the influence of 5,7-DHT lesioning on the down-regulation of presynaptic α_2-adrenoceptors by desipramine and ECS in the study of Heal *et al.* (1990). Data reported by Heal *et al.* (1990) showed that this 5,7-DHT lesion produced a 77% reduction of brain 5-HT, with minimal effects on noradrenaline or dopamine levels, and produced a marked supersensitivity of 5-HT$_2$-mediated head-twitch behaviour. However, as shown in the present investigation, the denervation procedure did not alter clonidine-induced mydriasis or its reduction by repeated administration of either desipramine or ECS. The lack of a permissive role for 5-HT-containing neurones in the adaptation of postsynaptic α_2-adrenoceptors contrasts sharply with their involvement in the down-regulation of both presynaptic α_2- and β-adrenoceptors. Thus, clonidine-induced hypoactivity, which is an index of presynaptic α_2-adrenoceptor function in the brain (Heal *et al.*, 1989b), is attenuated by repeated administration of desipramine (Heal *et al.*, 1983) and ECS (Heal *et al.*, 1981). When 5-HT-containing neurones were destroyed by the 5,7-DHT lesioning protocol employed in this study, this procedure prevented the effects of the former, but not the latter, antidepressant treatment (Heal *et al.*, 1990). Similarly, 5,7-DHT lesioning has also been reported to inhibit the down-regulation of β-adrenoceptors by a variety of antidepressants, including desipramine and ECS (Brunello *et al.*, 1982; Nimgaonkar *et al.*, 1985). More recently, Asakura *et al.* (1987) have refined this hypothesis by arguing that 5-HT-containing neurones do not in fact play a permissive role in β-adrenoceptor down-regulation process, but

they are essential for the prolonged maintainance of this adaptive response.

Overall, the results presented demonstrate that intact noradrenergic neurones are essential for the attenuation of clonidine-induced mydriasis by desipramine, but not ECS. In addition, 5-HT-containing neurones do not play a permissive role in the antidepressant-induced adaptation of this postsynaptic α_2-adrenoceptor-mediated response.

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Analgesic doses of morphine do not reduce noxious stimulus-evoked release of immunoreactive neurokinins in the dorsal horn of the spinal cat

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1 Antibody microprobes were used to detect immunoreactive neurokinin A release in the dorsal spinal cord of barbiturate-anaesthetized spinal cats.

2 Noxious mechanical stimulation of the ipsilateral hind paw and electrical stimulation (suprathreshold for unmyelinated primary afferent fibres) of the ipsilateral tibial nerve evoked immunoreactive neurokinin A release.

3 Systemic morphine, 5 mg kg^{-1}, i.v., did not block immunoreactive neurokinin A release in response to these stimuli.

4 Subsequent naloxone administration, 0.5 mg kg^{-1}, i.v., did not alter this stimulus-evoked release.

5 Basal levels of immunoreactive neurokinin A were unaltered by morphine or naloxone.

6 These results suggest that the analgesic effects of morphine at the spinal cord level are not brought about by activation of presynaptic opiate receptors on neurokinin A containing afferent terminals.

Keywords: Neurokinins; morphine; naloxone; antibody microprobes; cat spinal cord.

Introduction

Substance P and neurokinin A, two members of the tachykinin family of neuropeptides, extensively coexist in the small diameter dorsal root ganglion cells of the rat (Dalsgaard *et al.*, 1985). Both tachykinins have been proposed as important in the transmission of information from nociceptive afferents to spinal or brainstem neurones (Otsuka & Yanagisawa, 1988; Fleetwood-Walker *et al.*, 1990). Studies of the release of these compounds in the spinal cord by use of antibody microprobes have shown both tachykinins to be released in response to a similar range of peripheral noxious stimuli (Duggan *et al.*, 1988b; 1990; Hope *et al.*, 1990a; Schaible *et al.*, 1990) although important differences in noxious stimuli release patterns have also been identified. Noxious stimuli producing detectable tissue damage caused greater release of immunoreactive substance P in the dorsal horn, than noxious, non-damaging stimuli. Noxious cutaneous stimuli also released immunoreactive neurokinin A, but potentiation by tissue damage was not seen. Immunoreactive neurokinin A exhibited a remarkable spread beyond sites of release and persisted following an effective stimulus. In contrast a focal, non-persistent pattern was observed with immunoreactive substance P. These findings suggest that neurokinin A, unlike the relatively short-lived substance P, may not function as a conventional neurotransmitter, but might rather have a long term modulatory role on spinal cord systems.

Iontophoretic administration of morphine (Duggan *et al.*, 1977) and the μ opioid agonist [D-Ala^2 Me-Phe^4, Gly-ol^5] enkephalin (DAMGO) (Fleetwood-Walker *et al.*, 1988) in the region of the substantia gelatinosa selectively suppressed the transmission of impulses from nociceptive afferents to deeper neurones of the dorsal horn. The substantia gelatinosa is the major site of termination of unmyelinated cutaneous afferent fibres (Light & Perl, 1977). Since administration of naloxone in the substantia gelatinosa completely blocked the effects of analgesic doses of systemic morphine on deep neurones of the dorsal horn of the cat (Johnson & Duggan, 1981) it is likely that opiate receptors in the substantia gelatinosa are the major ones mediating the analgesic actions of morphine at the spinal level, whether administered systemically or intrathecally. One mechanism by which morphine could produce analgesia is by presynaptic inhibition of neurotransmitter release from nociceptive primary afferent terminals. In support of this hypothesis, morphine in high concentrations, when added to the superfusate of either a slice of the upper dorsal horn (Jessel & Iversen, 1977; Mauborgne *et al.*, 1987) or of the spinal cord *in vivo* (Yaksh *et al.*, 1980; Go & Yaksh, 1987) has been shown to reduce stimulus evoked release of immunoreactive substance P. However, systemically administered morphine at analgesic doses has failed to reduce release of immunoreactive substance P evoked by noxious stimulation (Kuraishi *et al.*, 1983; Morton *et al.*, 1990).

With the recognition that a peripheral noxious stimulus results in the central release of a number of neuroactive compounds including amino acids (Kangrga & Randic, 1990) and peptides (Duggan *et al.*, 1988b; 1990), a complete study of opiates and release from primary afferents is clearly a complex task. There is evidence that the arrival of impulses in nociceptive afferents produces not only fast transmission to fibres ascending to the brain but also long lasting events such as prolonged facilitation of flexor reflexes (Woolf, 1983; Woolf & Wall, 1986a; Cook *et al.*, 1987; Cervero *et al.*, 1988; Neugebauer & Schaible, 1988; Hoheisel & Mense, 1989; Hylden *et al.*, 1989). It has been proposed that neurokinin A (rather than substance P) is more important in these prolonged spinal events since, following release of immunoreactive neurokinin A, this peptide (or an extended form such as neuropeptide K) diffuses widely and persists in the spinal cord (Hope *et al.*, 1990a). Of note is the finding that in the rat, low doses of morphine suppressed the prolonged facilitation of spinal reflexes produced by nociceptive input, whereas higher doses were required to suppress fast transmission as measured by an unfacilitated nociceptive reflex (Woolf & Wall, 1986b). Such considerations indicate the need for a study of opiates and the central release of neurokinin A. In the present study therefore, the effects of analgesic doses of systemically administered morphine were examined on the noxious stimulus-evoked release of immunoreactive neurokinin A in the dorsal horn of the spinal cord in barbiturate-anaesthetized cats.

Methods

Preparation of antibody microprobes

Antibody microprobes were prepared as previously described (Duggan *et al.*, 1988a). Fine glass micropipettes, heat sealed at

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base and tip, were incubated in a 10% solution of aminopropyltriethoxysilane producing an even layer of a siloxane polymer bearing free amino-groups on the outer surface of the probes. Glutaraldehyde was then used to immobilize protein A (Sigma) onto this polymer; protein A then bound immunoglobulins present in an antiserum raised against the carboxyl terminus of neurokinin A (Peninsula). Although this antiserum is described as anti-neurokinin A, information from the manufacturer indicates it has 100% cross reactivity with neuropeptide K (an N-terminal extended form of neurokinin A), 70–80% cross-reactivity with neurokinin B, but negligible cross-reactivity with substance P. For purposes of the present experiments microprobes are described as detecting immunoreactive neurokinins.

The antiserum lyophylate contained salts from the suspending buffer which limited the possible concentration of resuspended antibody. A dilution of approximately 1:3000 was used. All protein binding outside the spinal cord took place in 5 μl glass capillaries containing the relevant solutions.

Animal preparation/recording/microprobe analysis

Experiments were performed on 8 cats anaesthetized initially with intraperitoneal sodium pentobarbitone 35 mg kg^{-1}, and maintained by continuous intravenous infusion of sodium pentobarbitone, 3 mg kg^{-1} h^{-1}. All animals were artificially ventilated with air and when necessary paralysed with gallamine triethiodide, 4 mg kg^{-1} h^{-1}. Blood pressure was measured continuously via a cannula in the left carotid artery and end tidal CO_2 levels were also monitored and maintained at 4%.

The lumbar spinal cord was exposed by dorsal laminectomy and the spinal cord transected at the level of T13/L1 following injection of 0.1 ml of 2% lignocaine (Astra). The lumbar dura mater was cut longitudinally at the level of L7-S1 and retracted laterally. The exposed cord was then covered with a thin layer of Ringer/agar. At sites of proposed microprobe insertion a small area of agar was removed to form a pool and the underlying pia-arachnoid removed with fine sterile forceps. Such areas were then continuously irrigated with sterile Ringer solution at 37°C. Microprobes were inserted into the spinal cord with stepping motor micromanipulators. The first probe in each new area was used to obtain extracellular recordings to identify the receptive fields, to light brushing of the skin, of neurones encountered along a microprobe track.

Peripheral noxious stimulation was provided either by applying 'crocodile' clips to the pads and interdigital skin of the ipsilateral hind paw in a cycle of 3 min on, 2 min off, for the duration of the stimulus or by electrical stimulation of the ipsilateral tibial nerve (groups of three 0.5 ms square wave pulses at 330 Hz, repeated at 10 Hz, amplitude 20 V). All microprobes were inserted 3 mm into the dorsal spinal cord which, in an adult cat, places the tips in the upper ventral horn. All probes remained in the spinal cord for 30 min following insertion.

Antibody microprobes demonstrate localized binding of an endogenous ligand by their subsequent failure to bind exogenous radiolabelled ligand. Following removal from the spinal cord, microprobes were washed for 15 min in cold phosphate buffered saline containing Tween-80 (0.1%) and then incubated for 24 h at 4°C in a phosphate buffered saline/azide solution of [^{125}I]-neurokinin A containing 0.5% bovine serum albumin diluted to give approximately 2000 c.p.m. μl^{-1}. Following this incubation, microprobes were again washed for 15 min in PBS-Tween with suction applied to the base of the microprobes to draw any labelled neurokinin A out of the tips. The tips were then broken off and fixed to a sheet of paper which was placed in an X-ray film cassette with a sheet of monoemulsion X-ray film (NMB-Kodak). Exposure times were typically in the order of 3–4 weeks reflecting the limited amounts of this antibody which could be bound to the probe surfaces. In each experiment 8 microprobes were used for *in vitro* tests of sensitivity. Four of these were incubated with 10^{-7} mol l^{-1} neurokinin A for 30 min at 37°C prior to incubation in [^{125}I]-neurokinin A. The other 4 were simply incubated with the radiolabelled neuropeptide.

The resulting X-ray film images were analysed with an image analysis system employing an Imaging Technology PC Vision Plus frame grabber board operating in a DCS 286e (AT-based) computer. A television (CCD) camera was used as described previously (Hendry *et al.*, 1988) to produce a transverse integration of optical density, with a resolution of 10 μm, along the length of each microprobe. These integrals were stored on a hard disk together with 32 coded values assigned to various experimental parameters for each microprobe. These codings allowed sorting of microprobes into groups meeting stated criteria. Images from these groups could then be averaged and the differences between control and test groups calculated and assigned levels of statistical significance. It should be emphasized that the spatial resolution of this method is 100 sites (each of 10 μm) per mm, and that events at each site are considered independently of others in the averaging of images and the calculation of group differences and their significances.

Drug dosage and experimental regimes

The drugs used were administered via an intravenous catheter into the right cephalic vein. Morphine was administered as a 10 mg ml^{-1} solution in physiological saline, naloxone as 1 mg ml^{-1} in physiological saline.

As previously demonstrated (Duggan *et al.*, 1990; Hope *et al.*, 1990a) the elevation of immunoreactive neurokinin levels in the dorsal spinal cord, following a discrete stimulus, persists for up to 1 h beyond stimulation. This persistence creates difficulties when testing a compound which may block release, since a series of pre-drug measurements of stimulus-evoked release may result in a build up of immunoreactive neurokinins in the dorsal horn such that any subsequent reduction in release brought about by the drug may not be detectable. Consequently, these experiments examined the basal levels of immunoreactive neurokinins in the absence of any stimulus and then administered morphine prior to measuring stimulus-evoked release of these compounds. Several groups have shown that in the spinal cat, doses of morphine of 0.3 to 4.0 mg kg^{-1} i.v. reduced the synaptic activation of dorsal horn neurones by peripheral noxious stimuli (Le Bars *et al.*, 1976a,b; Duggan *et al.*, 1980; Johnson & Duggan, 1981). The present experiments used morphine at levels just above the upper limit of this range (5.0 mg kg^{-1}, i.v.) since, with the protocol used, lack of effectiveness of the stimulus would be more readily detected than a partial reduction. A more significant test of the effect of morphine on immunoreactive neurokinin release came from the effects of subsequent naloxone administration. If stimulus evoked release of immunoreactive neurokinins is increased when naloxone is administered after morphine, this indicates that morphine had reduced release of these compounds. The above protocol is not optimal if morphine increases release of immunoreactive neurokinins. A series of experiments was therefore included in this study in which naloxone was administered prior to morphine in an attempt to block such an increase. Comparisons were also made with earlier studies on the effects of stimulus-evoked release of immunoreactive neurokinins in the absence of morphine (Hope *et al.*, 1990a,b).

Results

A total of 97 microprobes coated with antibodies to neurokinin A were inserted into the spinal cord. Additionally, 64 microprobes were used for concurrent *in vitro* tests. These consistently demonstrated suppression of the binding of [^{125}I]-neurokinin A by greater than 50% following incubation with neurokinin A 10^{-7} mol l^{-1}.

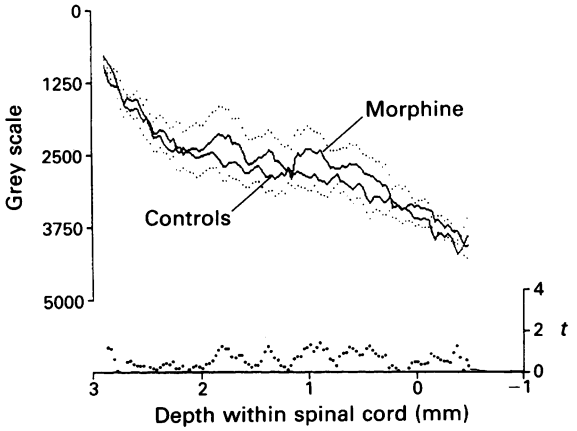

Figure 1 The effects of systemic morphine administration on resting levels of immunoreactive neurokinins in the dorsal horn. Controls: the mean image analysis of microprobes ($n = 11$) inserted into the dorsal spinal cord with no prior or concurrent drug administration and no prior to concurrent peripheral stimulation. Morphine: the mean image analysis of microprobes ($n = 7$) inserted into the dorsal spinal cord immediately following morphine ($5\,mg\,kg^{-1}$, i.v.) administration and in the absence of prior or concurrent peripheral stimulation. The analysis was performed with a resolution of 33 points per millimetre and with each group the continuous line has joined these points. The (+) s.e.mean at each point is plotted for the morphine group and the (−) s.e.mean at each point is plotted for the control group. The t values ($2t = P < 0.05$) derived from the differences between the means of the two groups are also shown.

Thirty-five probes were inserted 3 mm into the cord and left for 30 min without stimulation of either hind paw or the concurrent administration of any drug. These microprobes formed the basis for assessing possible effects of morphine or naloxone on the basal levels of immunoreactive neurokinins. The remaining 62 probes were inserted into the spinal cord in the presence of peripheral noxious stimuli and/or drug administration.

Effects of morphine and naloxone on basal levels of immunoreactive neurokinins

In four experiments, morphine was the first drug administered and in the remaining experiments naloxone was the first drug administered. Seven probes were inserted into the spinal cord in the absence of prior or concurrent noxious stimuli, and

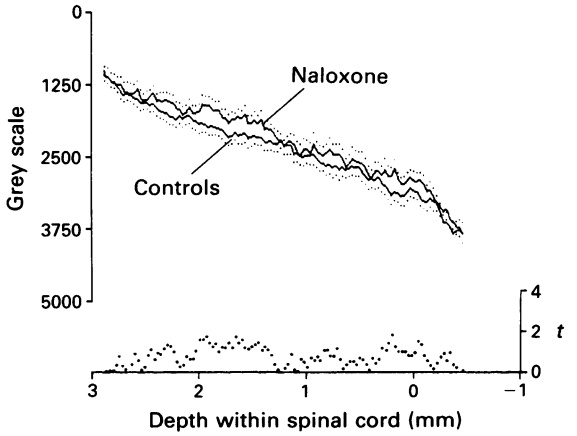

Figure 2 The effects of systemic naloxone administration on resting levels of immunoreactive neurokinins in the dorsal horn. Controls: the mean image analysis of microprobes ($n = 23$) inserted into the dorsal spinal cord with no prior or concurrent drug administration and no prior or concurrent peripheral stimulation. Naloxone: the mean image analysis of microprobes ($n = 13$) inserted into the dorsal spinal cord immediately following naloxone ($0.5\,mg\,kg^{-1}$, i.v.) administration and in the absence of prior or concurrent peripheral stimulation. S.e.means for the two groups and t values ($2t = P < 0.05$) derived from the differences of the means are shown as in Figure 1.

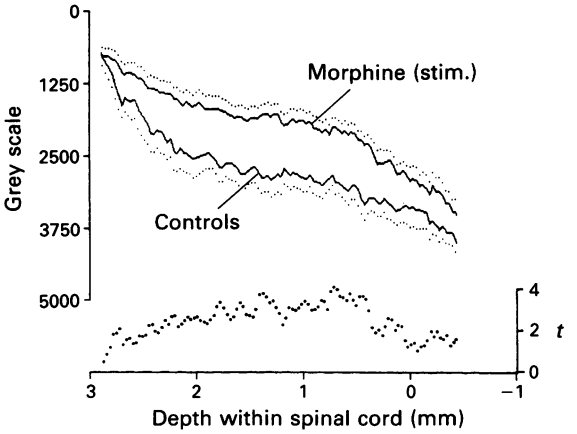

Figure 3 The effects of noxious stimulation of the ipsilateral hind paw, following systemic morphine administration on immunoreactive neurokinin levels in the dorsal horn. Controls: the mean image analysis of microprobes ($n = 11$) inserted into the dorsal spinal cord with no prior or concurrent drug administration and no prior or concurrent peripheral stimulation. Morphine (stim): the mean image analysis of microprobes ($n = 17$) inserted into the dorsal spinal cord following morphine ($5\,mg\,kg^{-1}$, i.v.) administration and during noxious stimulation of the ipsilateral hind paw. S.e.means for the two groups and t values ($2t = P < 0.05$) derived from the differences of the means are shown as in Figure 1.

within 1 min of morphine administration ($5\,mg\,kg^{-1}$, i.v.). In all cases, this dose of morphine was the first drug administered. The mean image analysis of these probes is illustrated in the upper trace of Figure 1. When this group is compared to the mean image analysis of microprobes inserted in the absence of prior peripheral stimulation in the same experiments (lower trace, 'controls', Figure 1), no significant differences are present as shown in the plot of the t statistics for the differences between these groups of microprobes.

Thirteen probes were inserted into the spinal cord in the absence of prior or concurrent noxious stimulation and within 1 min of naloxone administration ($0.5\,mg\,kg^{-1}$, i.v.). No prior morphine administration had occurred. The mean image analysis of this group of microprobes and the relevant (no stimulus) controls are shown in Figure 2. The plot of the t statistics derived from the differences between these groups shows that no significant differences are present.

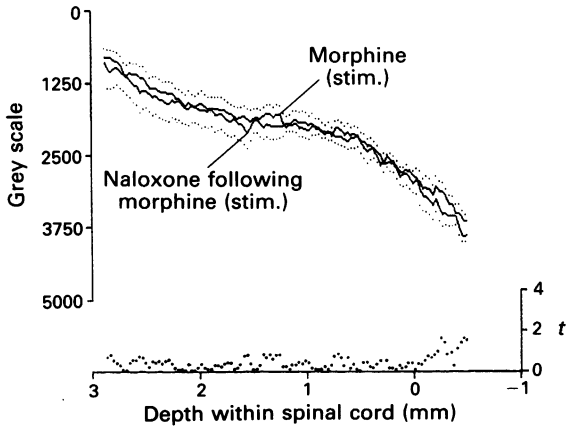

Figure 4 Effect of naloxone following morphine on noxious stimulus evoked immunoreactive neurokinin release. Morphine (stim): the mean image analysis of microprobes ($n = 17$) as described in Figure 3. Naloxone following morphine (stim): the mean image analysis of microprobes ($n = 10$) inserted into the dorsal spinal cord immediately following those illustrated in the morphine (stim) group, and after naloxone ($0.5\,mg\,kg^{-1}$, i.v.) administration during noxious stimulation of the ipsilateral hind paw. S.e.means for the two groups and t values ($2t = P < 0.05$) derived from the differences of the means are shown as in Figure 1.

An additional 10 probes were inserted into the spinal cord in the absence of prior or concurrent noxious stimulation and within 1 min of morphine administration (5 mg kg^{-1}, i.v.) but in this case morphine immediately followed naloxone (0.5 mg kg^{-1}, i.v.) administration. Again, when compared with the relevant control group no significant differences were found.

In summary, neither morphine nor naloxone, administered in the absence of peripheral noxious stimulation, produced significant changes in detectable levels of immunoreactive neurokinins in the dorsal spinal cord. A combination of these two drugs also failed to alter significantly basal levels of immunoreactive neurokinins.

Effect of morphine on stimulus-evoked immunoreactive neurokinin release

Seventeen microprobes were inserted into the dorsal spinal cord within 90 min of morphine administration (5 mg kg^{-1}, i.v.) and with concurrent noxious stimulation of the ipsilateral hind paw. Noxious pinch and electrical stimulation of unmyelinated primary afferents have previously been shown to be adequate stimuli for evoking immunoreactive neurokinin release in the dorsal spinal cord (Duggan *et al.*, 1990; Hope *et al.*, 1990a). Following morphine administration, noxious stimulus dependent immunoreactive neurokinin release was seen in the present experiments in a zone extending from the spinal cord surface to approximately 1.3 mm into the dorsal grey matter as shown in Figure 3. Although the increases in the levels of immunoreactive neurokinins in the dorsal horn produced by the stimulus appeared comparable to those produced by comparable stimuli in previous studies in which morphine had not been administered (Duggan *et al.*, 1990; Hope *et al.*, 1990a) a small inhibition of release by morphine could only be assessed by the subsequent administration of naloxone.

Eleven probes were inserted into the dorsal spinal cord within 1 min of naloxone (0.5 mg kg^{-1}, i.v.) given after morphine and during peripheral noxious stimulation. These probes were inserted after those represented in Figure 3, i.e. immediately after the period of stimulation which followed morphine administration. Figure 4 shows that naloxone had no effect on the stimulus-evoked levels of immunoreactive neurokinins in the dorsal horn which were observed following morphine. This lack of effect of naloxone suggests that the dosage of morphine used prior to naloxone had not altered the release of immunoreactive neurokinins.

Discussion

When the results of the present experiments are considered with those of Kuraishi *et al.* (1983) and Morton *et al.* (1990), there is little evidence that analgesic doses of opiates administered systemically reduce tachykinin release (either neurokinin A or substance P) from the central terminals of nociceptors. Since such results are at variance with the conclusions of experiments which have examined the effect on substance P release of topically applied opiates, these differences require comment.

Inhibition of substance P release by opiates was first shown by Jessell & Iversen (1977) in a slice preparation of rat trigeminal nucleus. The stimulus used, however, was 47 mM potassium in the perfusate. This will depolarize virtually all neural structures in the slice, and hence the source of substance P is uncertain. Furthermore, the concentration of morphine used was high (10^{-5} M). Using the push-pull cannula technique on rabbit spinal cord *in vivo*, Hirota *et al.* (1985), demonstrated a reduction in the release of substance P following noxious cutaneous stimulation with morphine (10^{-5} M) in the perfusate. Further support for the proposal that opiates impair substance P release from the central terminals of nociceptors came from the experiments of Yaksh *et al.* (1980) and Go & Yaksh (1987), in which morphine (10^{-4} M) added to a superfusate of intact cat spinal cord *in vivo* reduced the release of substance P into the perfusate following electrical stimulation of unmyelinated primary afferents.

Mauborgne *et al.* (1987), however, found that while the μ-receptor agonist DAMGO (10^{-5} M) increased both high potassium and capsaicin induced release of substance P from dorsal horn slices, the δ-receptor agonist [D-Thr^2]-Leu-enkephalin-Thr (DTLET) (3×10^{-6} M) reduced release in the same preparation. The κ-agonist, U50488H (0.5–50 $\times$ 10^{-6} M), did not affect release of substance P (Hamon *et al.*, 1988; Pohl *et al.*, 1989).

It should be noted that the concentrations of drugs used in all of the above studies were high and that no group has found that systemic analgesic doses of morphine reduce substance P release in the dorsal horn of spinal animals. Thus, Kuraishi *et al.* (1983), using push pull canulae in the dorsal horn of the spinal rabbit observed that systemic morphine (10 mg kg^{-1}) failed to reduce immunoreactive substance P release from noxious mechanical stimulation. Using antibody microprobes, Morton *et al.* (1990) failed to observe any effect of morphine given in two dose ranges (1–6 mg kg^{-1} and 10–20 mg kg^{-1}) on immunoreactive substance P release in the dorsal horn of the spinal cat following noxious mechanical or thermal cutaneous stimulation.

Since studies which have observed effects on tachykinin release have used relatively high concentrations of opiates, is the same true of experiments which have administered opiates microiontophoretically in the substantia gelatinosa and hence may have over-emphasized the importance of effects near the first central synapse of nociceptors? Although concentrations in microiontophoretic experiments are unknown, the finding that naloxone administered in the substantia gelatinosa decreased the effects of systemic morphine, 4 mg kg^{-1}, on deeper neurones (Johnson & Duggan, 1981) does support the importance of opiate receptors in the substantia gelatinosa in mediating the action of systemic morphine on spinal neurones.

When γ-aminobutyric acid acts presynaptically on the central terminals of large diameter muscle afferents, the resultant depolarization is associated with reduced transmitter release and an increased excitability when tested with an adjacent stimulating microelectrode (Curtis *et al.*, 1980). Administering opioids near the central terminals of nociceptors in the substantia gelatinosa however, has resulted in decreased electrical excitability (Sastry, 1980; Carstens *et al.*, 1987), a result not consistent with the well-established mechanism of presynaptic inhibition in the spinal cord. A different mechanism of reducing transmitter release might be if calcium entry with each invading impulse were to be reduced through a shortening of action potential duration. Such a shortening has been observed with micromolar concentrations of opioids acting on cultured dorsal root ganglion neurones (Werz & Macdonald, 1982; Shen & Crain, 1989) but lower (nanomolar) concentrations had the opposite effect (Shen & Crain, 1989). Support for a postsynaptic effect of opioids on intrinsic neurones of the substantia gelatinosa has come from Yoshimura & North (1983) who observed a hyperpolarization of such cells in a slice preparation, and Sastry & Goh (1983) who found many to be excited *in vivo* by iontophoretically administered morphine. The inconsistency in these reports however has not been explained.

Recently Lombard & Besson (1989) have tried to assess the relative importance of pre- and postsynaptic actions of morphine by recording the firing of dorsal horn neurones in decerebrate spinal rats with intact dorsal roots (and an induced peripheral arthritis) and comparable cells in non-arthritic animals with sectioned dorsal roots. Morphine (2 mg kg^{-1}, i.v.) depressed the spontaneous firing of neurones in both preparations but a greater effect was observed in the arthritic animals with intact dorsal roots. Such a result indicates an action by opiates on the pathway from primary afferent terminals to the neurones studied but cannot distinguish between effects on terminals and effects on interneurones interposed between such terminals and the neurones studied.

It has been the conclusion of studies which have observed reductions in tachykinin release with high concentrations of opiates that a presynaptic action on the central terminals of nociceptors is an important component of opiate analgesia (Yaksh & Noueihed, 1985). The present experiments considered with those of Kuraishi *et al.* (1983) and Morton *et al.* (1990) suggest that such an action does not occur significantly with tachykinin releasing fibres in the spinal cord following clinically relevant doses of morphine.

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Mechanisms controlling caffeine-induced relaxation of coronary artery of the pig

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1 We studied the effects of caffeine on coronary artery smooth muscle of the pig by measuring changes in isometric tension, cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and transmembrane potential.

2 In the absence of tone, caffeine induced a concentration-dependent transient contraction of coronary artery strips, followed by sustained relaxation. Simultaneously with the relaxation, caffeine, 25 mM, hyperpolarized the smooth muscle cells by 7.7 ± 0.9 mV.

3 Caffeine caused a concentration-dependent relaxation of strips precontracted with 10^{-5} M acetylcholine (ACh). A supramaximal relaxing concentration of 25 mM caffeine produced an additional transient increase in $[Ca^{2+}]_i$ on the Ca^{2+} plateau of ACh tonic contraction, which was followed by a decrease in $[Ca^{2+}]_i$ to a level slightly below the basal concentration. This relaxation was accompanied by a hyperpolarization of 7.3 ± 0.9 mV.

4 KCl 120 mM (high K^+) contracted the strips with a concomitant depolarization of 38.6 ± 1.6 mV and sustained increase in $[Ca^{2+}]_i$. Caffeine caused a concentration-dependent relaxation of high K^+-induced contraction. Caffeine, 25 mM, decreased the Ca^{2+} plateau to a level that remained above the basal concentration of Ca^{2+} but did not change the membrane potential.

5 When strips were placed in a Ca^{2+}-free medium with EGTA 2 mM and, in addition, ACh was applied successively three times, both intracellular and extracellular mobilizable Ca^{2+} pools were depleted. In these conditions, phorbol 12,13 dibutyrate (PDBu) 10^{-7} M and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 10^{-5} M contracted the strips. Caffeine (25 mM) inhibited these contractions with no change in $[Ca^{2+}]_i$.

6 Forskolin, 3×10^{-7} M, inhibited ACh induced-contraction but did not affect those induced by PDBu.

7 In conclusion, these results show that caffeine has multiple cellular effects. During caffeine-induced relaxation, $[Ca^{2+}]_i$, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) content and membrane potential are modified. The findings suggest, however, that these effects are secondary, and that caffeine acts mainly by another unknown mechanism, possibly involving a direct inhibition of the contractile apparatus.

Keywords: Caffeine, Ca^{2+}, smooth muscle, Ca^{2+} independent, coronary artery

Introduction

Caffeine has been widely used as a pharmacological tool to study contraction-relaxation cycles in smooth muscle cells. Caffeine causes transient contractions in smooth muscles by releasing Ca^{2+} from intracellular or extracellular stores (Ito & Kuriyama 1971; Sunano & Miyazaki, 1973; Casteels *et al.*, 1977; Itoh *et al.*, 1982; Karaki *et al.*, 1987; Matsumoto *et al.*, 1990). In addition to these contractile effects, caffeine has potent relaxing activities on precontracted smooth muscles (Leijten & van Breemen, 1984; Sato *et al.*, 1988; Ahn *et al.*, 1988). The mechanisms of these relaxations are not completely understood as caffeine has multiple effects on intracellular machinery: inhibition of Ca^{2+} influx (Leijten & van Breemen, 1984; Martin *et al.*, 1989), increase in Ca^{2+} extrusion (Ahn *et al.*, 1988), increase in cytosolic adenosine 3′,5′-cyclic monophosphate (cyclic AMP) by the inhibition of phosphodiesterase (Polson *et al.*, 1978; Fredholm *et al.*, 1979; Bray *et al.*, 1989). Cyclic AMP increases Ca^{2+} uptake in sarcoplasmic reticulum (Saida & van Breemen, 1984), inhibits myosin light chain kinase activity (Conti & Adelstein, 1980) and decreases Ca^{2+} influx (Abe & Karaki, 1988). All these mechanisms implicate Ca^{2+}. Indeed, a large number of contractile agonists mobilize extracellular or intracellular Ca^{2+}, but contractions independent of Ca^{2+} have been described, notably via protein kinase C activation with phorbol esters (Chatterjee & Tejada, 1986; Singer & Baker, 1987), prostaglandins (Bradley & Morgan, 1987; Heaslip & Sickels, 1989) or with okadaic acid (Ozaki *et al.*, 1987; Hirano *et al.*, 1989).

Our goal was to evaluate more fully the mechanisms by which caffeine induces vascular relaxation. During caffeine-induced relaxation, isometric tension, changes in $[Ca^{2+}]_i$ and smooth muscle cell membrane potential were measured. Caffeine was tested under conditions in which contractions were accompanied by an increase in $[Ca^{2+}]_i$ without changes in membrane potential (acetylcholine (ACh)-induced contraction), on contractions accompanied by an increase in $[Ca^{2+}]_i$ and depolarization (high K^+-induced contraction), and on contractions independent of Ca^{2+} (phorbol ester-induced contraction).

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Methods

Pharmacological experiments

The anterior left descending branch of pig coronary artery was taken from freshly killed pigs at the local slaughterhouse. The lumen was quickly rinsed with ice cold oxygenated Krebs solution of the following composition (mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.8, glucose 10.1 and gassed with 95% O_2 : 5% CO_2 at pH 7.4. The 120 mM KCl (high K^+) solution was identical except for an equimolar substitution of KCl for NaCl. The artery was cut longitudinally and the endothelium was removed by gently rubbing with a cotton tip. Segments were cleaned of all adherent fat and connective tissue. Tension was measured in a 85 μl tissue bath (Mastrangelo & Mathison, 1983) by use of two silk threads tied to the extremities of the strip. One extremity was attached to the bottom of the bath and the other to a Grass force displacement transducer (FTO3C). Changes in isometric tension were amplified (Lectromed 3559) and recorded on a chart paper with polygraphs (W + W Electronics). Strips were continuously superfused with oxygenated Krebs solution (1.25 ml min^{-1}) maintained at 37°C. The muscles were stretched up to 10 mNewton (mN) and allowed

to stabilize for about 1 h. This tension was readjusted to 10 mN during stabilization and was taken as the baseline.

Cytosolic free Ca^{2+} measurements

Segments of about 5 cm long were cut longitudinally and stretched over a tissue chamber (20 μl). The endothelium and the adventitia were carefully removed at the place of observation which corresponded to the surface of the chambers. Here the strip was 215 ± 16 μm thick ($n = 15$). The tissue was loaded with Fura-2 AM (10 μM), the acetoxymethyl ester form of Fura-2 (Grynkiewicz *et al.*, 1985), for 2 h in a HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid)-buffered solution containing (mM): NaCl 145, KCl 5, $CaCl_2$ 1, $MgSO_4$ 0.5, NaH_2PO_4 1, HEPES 20 and glucose 10.1 at pH 7.4; 37°C. The tissue was then rinsed for 1 h in the solution used for pharmacological experiments (described above) but with a modified gas mixture of 75% N_2; 20% O_2 and 5% CO_2, since high O_2 concentrations greatly reduce the intensity of Fura-2 fluorescence (Becker & Fay, 1987). The artery was then fixed in the chamber to reduce movement and continuously perfused (1.5 ml min^{-1}). All changes in perfusate were achieved without the introduction of bubbles which would have produced artefacts. Furthermore, continuous perfusion ensured that drug concentrations remained stable and removed the dye from the extracellular fluid. The fluorescence emitted by the cells corresponded to the field of the lens (magnification 10×) and was observed through a quartz lamella with a Nikon inverted microscope (Diaphot) equipped with a P1 Photometer. We used dual excitation wavelengths of 340 and 380 nm which were changed by sliding manually the double filter holder about every minute and more rapidly when the changes in $[Ca^{2+}]_i$ occurred. As in pig coronary artery changes in $[Ca^{2+}]_i$ during contraction-relaxation cycles are slow, this method permitted us to evaluate indirectly absolute changes in $[Ca^{2+}]_i$. A neutral density reduction filter was placed in front of the 380 nm excitatory filter to obtain roughly the same fluorescence intensity as in the 340 nm trace. A continuous control recording was made separately for each of the two excitatory wavelengths. Changes in fluorescence were recorded on a W + W Electronics recorder and the $[Ca^{2+}]_i$ levels were estimated by calculating the ratio of the emission at 510 nm following excitation at 340 and 380 nm (Grynkiewicz *et al.*, 1985). The ratio was calculated each time the filters were alternated. The advantage of the dual excitation ratio method is that it corrects for instrument artefacts and reduces problems associated with photobleaching (Hall *et al.*, 1988).

Electrophysiological experiments

Mechanical tension and transmembrane potential were measured simultaneously. The endothelium was removed as described previously. Strips were incubated in a perspex tissue bath, continuously perfused with oxygenated Krebs solution (1.25 ml min^{-1}) maintained at 37°C. One end of each strip was pinned onto a silicon rubber (Silgard) surface, intimal side facing up. The other end was attached horizontally to the force transducer. The strip was stretched to a tension of 10 mN and allowed to stabilize for 30 min, during which time tension was readjusted to 10 mN. The membrane potential was measured with conventional glass microelectrodes (80 MΩ) filled with 3 M KCl. Cells near the pins were impaled to reduce movement artefacts.

Drugs

Fura-2 acetoxymethyl ester, acetylcholine, prostaglandin $F_{2\alpha}$, phorbol 12,13 dibutyrate (PDBu) and forskolin were from Sigma. Fura-2 AM was dissolved in dimethyl sulphoxide (DMSO) and stored frozen at −20°C. Final concentration of DMSO never exceeded 1%. Prostaglandins and PDBu were dissolved in ethanol.

Data and analysis

Results are expressed as means ± s.e.mean. Comparisons of the results were made by Student's unpaired t test; $P < 0.05$ was accepted as significantly different.

Results

Effects of caffeine on basal tension

In the absence of tone, caffeine induced a concentration-dependent transient contraction of coronary artery strips that was associated with a transient increase in $[Ca^{2+}]_i$ (Figure 1). The contraction was followed by relaxation which was maximal at a concentration of 10^{-3} M. Simultaneously with the relaxation, 25 mM caffeine hyperpolarized the cells: the membrane potential was −43.7 ± 2.3 mV ($n = 3$) before, and −51.3 ± 1.5 mV ($n = 3$) after treatment with caffeine (Figure 1).

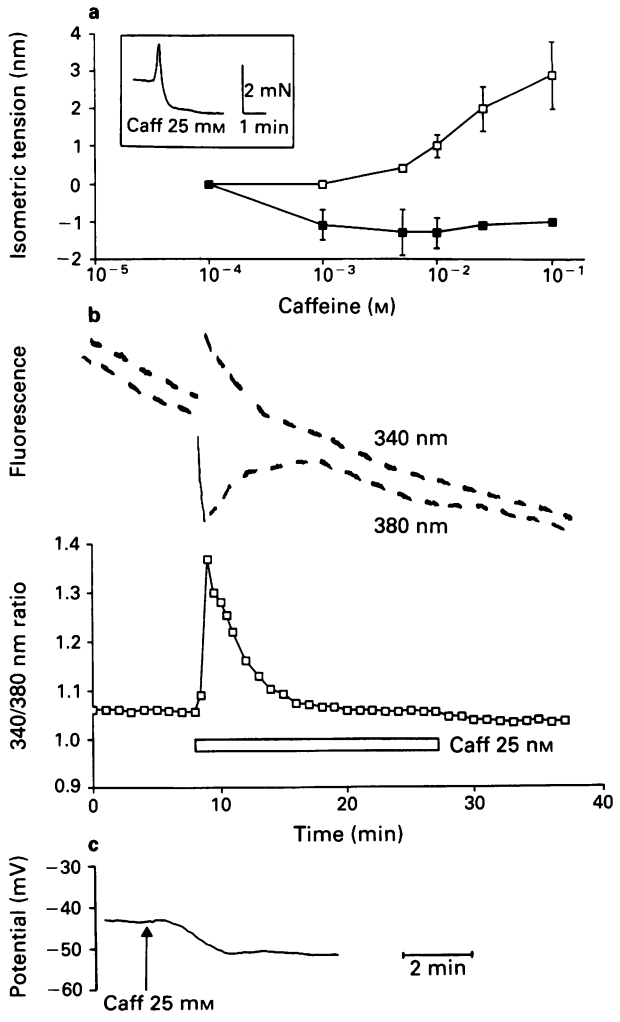

Figure 1 (a) Concentration-response curve of caffeine on the isometric tension of resting pig coronary artery strips. The zero value was related to the basal tension. Positive values represent contractions (□) and negative values represent relaxation (■) in relation to the basal tension. Points represent the mean and vertical bars the s.e.mean of 8 observations. The inset shows an original recording of the effect of 25 mM caffeine (Caff 25 mM). (b) Fluorescence signals of Fura-2 loaded strips were recorded at two excitation wavelengths, 340 and 380 nm. The breaks of the traces corresponded to the shifts of the excitation filters when they were changed from 340 to 380 nm. The ratio (340/380 nm) was calculated at each break and corresponded to an estimation of the changes in $[Ca^{2+}]_i$. (c) Recording of the smooth muscle cell transmembrane potential with conventional glass microelectrodes. These records are representative of 3 to 8 experiments.

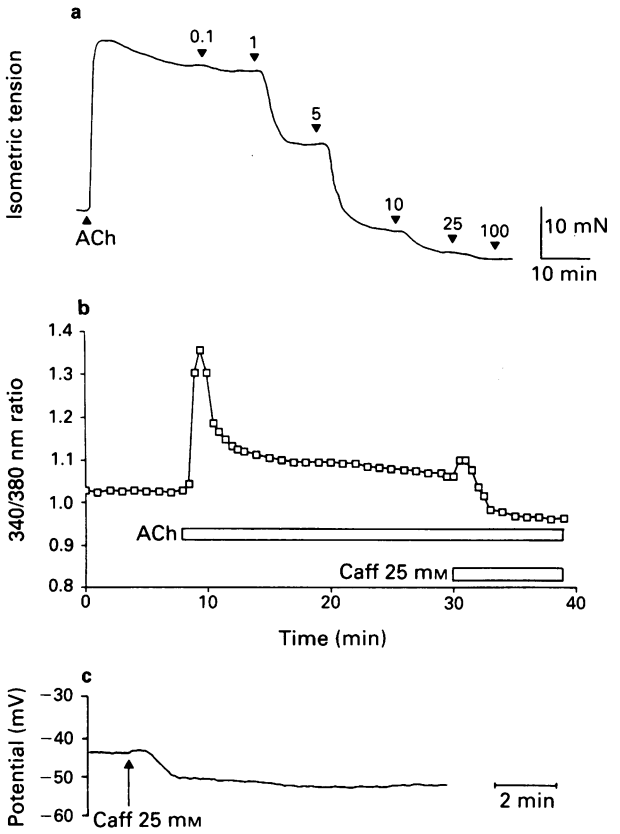

Figure 2 Effects of caffeine on strips precontracted with acetylcholine (ACh) 10^{-5} M. (a) A concentration-response curve with concentrations expressed in mM. (b) Estimation of $[Ca^{2+}]_i$ changes (ratio 340/380 nm). (c) A recording of the smooth muscle cell transmembrane potential. These records are representative of 4 to 8 experiments.

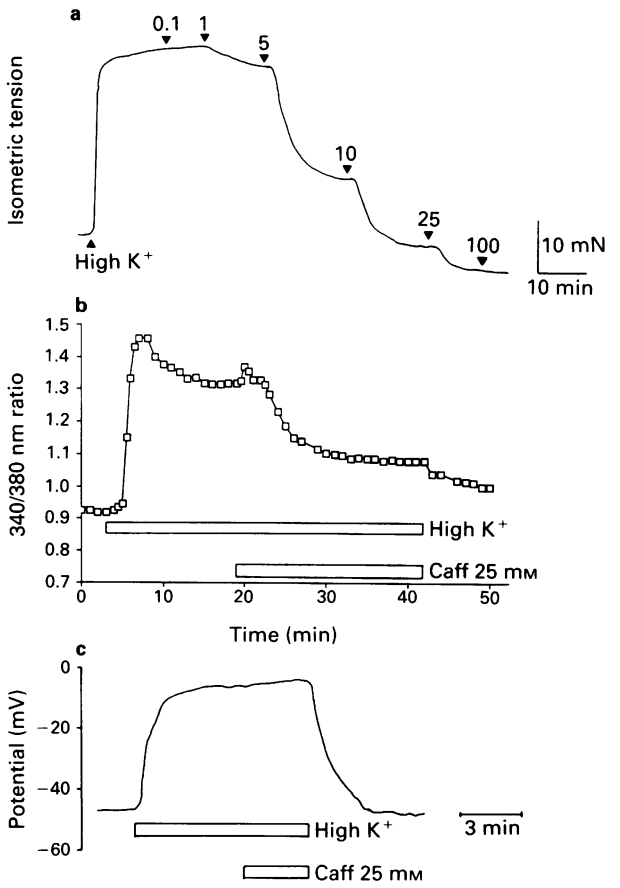

Figure 3 Effects of caffeine on strips precontracted with 120 mM KCl (High K^+). (a) A concentration-response curve with concentrations expressed in mM. (b) Estimation of $[Ca^{2+}]_i$ changes (ratio 340/380 nm). (c) A recording of the smooth muscle cell transmembrane potential. These records are representative of 7 to 8 experiments.

Effects of caffeine on acetylcholine-induced contractions

ACh (10^{-5} M) induced a phasic contraction followed by a sustained tonic contraction (Figure 2). The $[Ca^{2+}]_i$ was maintained slightly above the basal concentration during the tonic contraction. ACh contracted the strip without changing the membrane potential (data not shown). When applied during the ACh-induced contraction, caffeine caused a concentration-dependent relaxation. A supramaximal relaxing concentration of caffeine (25 mM) produced an additional transient increase in $[Ca^{2+}]_i$ on the Ca^{2+} plateau and this was followed by a decrease in $[Ca^{2+}]_i$ to a level slightly below the basal concentration. This was accompanied by hyperpolarization of the cells from -45.8 ± 1.7 mV ($n = 4$) before to -53.0 ± 1.1 mV ($n = 4$) after treatment with caffeine (Figure 2). Moreover, when 25 mM caffeine was applied simultaneously with 10^{-5} M ACh, only a phasic contraction was obtained. However, preincubation with 25 mM caffeine for 30 min inhibited both phasic and sustained contractions induced by ACh (data not shown).

Effects of caffeine on high K^+-induced contractions

High K^+-induced contraction was associated with a sustained increase in $[Ca^{2+}]_i$ and a depolarization of 38.6 ± 1.4 mV ($n = 7$) (Figure 3). Caffeine induced a concentration-dependent relaxation of high K^+-induced contraction. At a supramaximal concentration, caffeine (25 mM) induced an additional transient increase in $[Ca^{2+}]_i$ which then fell and stabilized above the basal concentration but did not alter the membrane potential (Figure 3). When caffeine was applied simultaneously with high K^+, the depolarization obtained was similar to that obtained in the absence of caffeine (data not shown).

However, when 25 mM caffeine was applied simultaneously with high K^+, only a transient contraction was observed. Moreover, preincubation with 25 mM caffeine for 30 min totally inhibited high K^+-induced contractions (data not shown).

Caffeine was less potent in relaxing strips contracted with high K^+ than with ACh (Figure 4) but complete relaxation was obtained in each case with 25 mM caffeine.

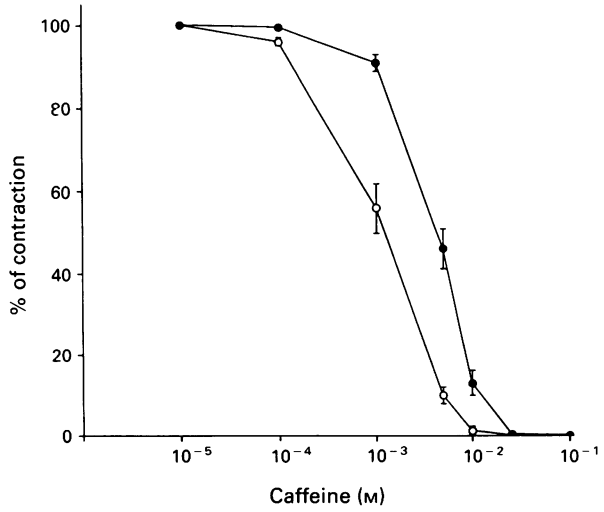

Figure 4 Concentration-response curve of the inhibitory effect of caffeine on strips precontracted with acetylcholine 10^{-5} M (○, $n = 8$) or KCl 120 mM (●, $n = 8$).

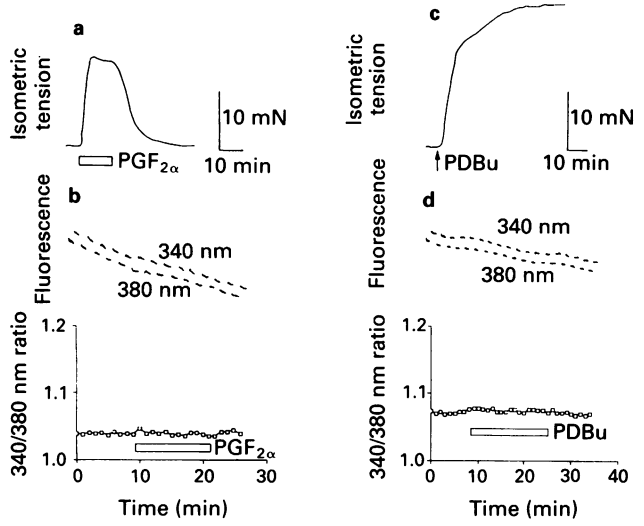

Figure 5 Effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 10^{-5} M (a, b) and phorbol 12,13 dibutyrate 10^{-7} M (PDBu) (c, d) on isometric tension and $[Ca^{2+}]_i$ (ratio 340/380 nm).

Effects of caffeine on Ca^{2+}-independent contractions

PDBu (10^{-7} M) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10^{-5} M) contracted the strips without changing $[Ca^{2+}]_i$ (Figure 5) and 25 mM caffeine inhibited these contractions completely. When the strips were placed in a Ca^{2+}-free solution containing EGTA (2 mM) and ACh was applied successively three times,

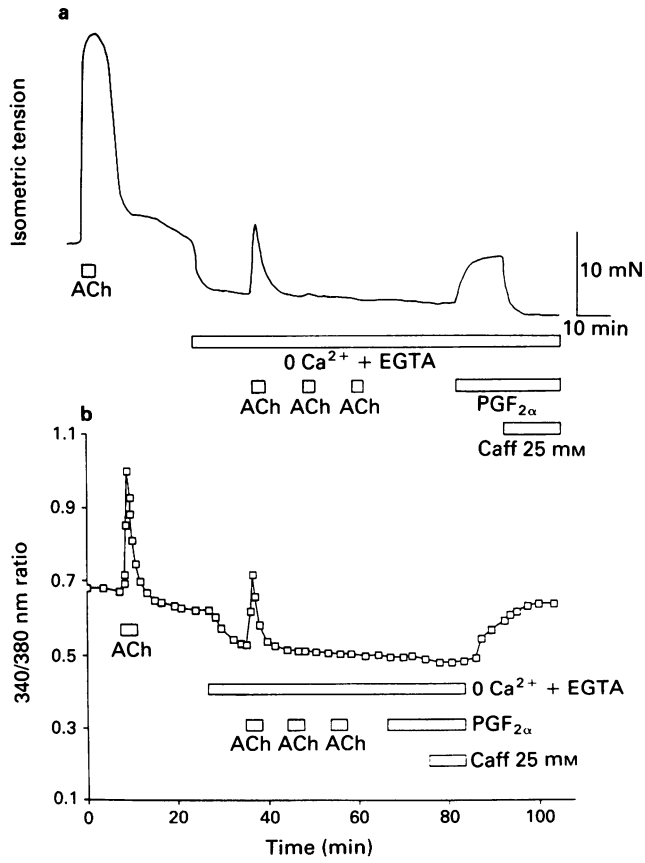

Figure 6 (a) Effects of caffeine (Caff) on isometric tension under conditions in which intracellular and extracellular mobilizable Ca^{2+} pools were depleted by applying acetylcholine (ACh) 10^{-5} M three times in succession in a Ca^{2+}-free EGTA (2 mM)-containing medium on strips precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 10^{-5} M. (b) Estimation of $[Ca^{2+}]_i$ changes (ratio 340/380 nm). These records are representative of 4 to 8 experiments.

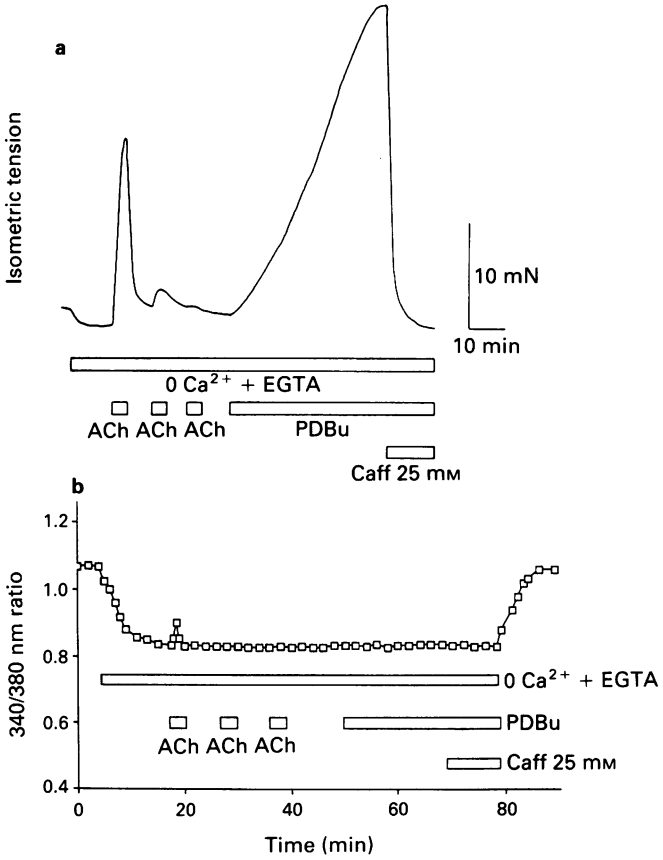

Figure 7 (a) Effects of caffeine (Caff) on isometric tension under conditions in which intracellular and extracellular mobilizable Ca^{2+} pools were depleted by applying acetylcholine (ACh) 10^{-5} M three times in succession in a Ca^{2+}-free EGTA (2 mM)-containing medium on strips precontracted with phorbol 12,13 dibutyrate (PDBu) 10^{-7} M. (b) Estimation of $[Ca^{2+}]_i$ changes (ratio 340/380 nm). These records are representative of 4 to 8 experiments.

both extracellular and intracellular mobilizable Ca^{2+} pools were depleted since ACh was no longer able to contract the strips and $[Ca^{2+}]_i$ was lower than the initial basal concentration (Figure 6). Under these conditions of Ca^{2+} depletion, $PGF_{2\alpha}$-induced contraction was reduced to $17 \pm 1\%$ ($n = 8$) of that obtained with the presence of extracellular Ca^{2+} (Figure 6). PDBu contracted the strips in the Ca^{2+}-free solution to the same degree of tension as that obtained in Ca^{2+}-containing solution, but contraction developed less rapidly (Figure 7). Under these conditions of Ca^{2+} depletion, caffeine (25 mM) inhibited $PGF_{2\alpha}$-induced contraction by $120 \pm 7\%$ ($n = 8$) and PDBu-induced contraction by $99 \pm 1\%$ ($n = 8$).

Effects of forskolin on acetylcholine and phorbol 12,13 dibutyrate-induced contractions

Forskolin 3×10^{-7} M, relaxed ACh (10^{-5} M)-induced contractions by $97 \pm 2\%$ ($n = 8$) in pig coronary artery but did not significantly affect PDBu-induced contractions, $P < 0.01$ ($n = 8$). Even at a concentration of 3×10^{-6} M, forskolin had no effect on PDBu-induced contractions (data not shown).

Discussion

Caffeine exhibited a rapid Ca^{2+}-dependent transient contraction followed by a sustained relaxation in pig coronary artery strips. ACh- and high K^+-induced contractions were inhibited by caffeine with no additional transient contraction and a lag before relaxation. This difference might be explained by the fact that in the latter case the contractions were already

maximal and caffeine was not able to induce a further contraction.

ACh-induced contraction was associated with a transient increase in $[Ca^{2+}]_i$ which was followed by a decrease to a plateau above the basal concentration but no change in membrane potential occurred. As caffeine hyperpolarized ACh-contracted strips and lowered $[Ca^{2+}]_i$, it is possible that hyperpolarization closed Ca^{2+} channels and led to a diminution of $[Ca^{2+}]_i$ resulting in relaxation. Indeed Martin *et al.* (1989) showed that caffeine inhibits Ca^{2+} influx by interacting with voltage-dependent Ca^{2+} channels. Hyperpolarization of pig coronary smooth muscle has already been observed in the relaxation induced by endothelium derived relaxing factors (Beny *et al.*, 1986; 1987; Beny & Brunet, 1988) and β-adrenoceptor agonists (Ito *et al.*, 1979). It is likely, however, that hyperpolarization is not essential since in the case of high K^+-induced contraction, where hyperpolarization was not possible, caffeine continued to produce relaxation. Nevertheless, in conditions in which hyperpolarization can occur, it is likely that closure of voltage-sensitive Ca^{2+} channels contributes to the relaxant action of caffeine.

Smooth muscle relaxation is believed to occur by a decrease in $[Ca^{2+}]_i$ and a dephosphorylation of myosin light chain (Gerthoffer & Murphy, 1983). Caffeine abolished the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ACh, but only partially reversed that induced by high K^+. Surprisingly, under both conditions, complete relaxation was induced by caffeine. Moreover, caffeine completely relaxed contractions induced by $PGF_{2\alpha}$ or PDBu under conditions of extracellular and intracellular Ca^{2+} depletion where the $[Ca^{2+}]_i$ remained below the basal concentration. Consequently, decrease in $[Ca^{2+}]_i$ is not a necessary prerequisite for caffeine-induced relaxation of pig coronary smooth muscle. It is likely, however, that under conditions where caffeine does reduce $[Ca^{2+}]_i$, this contributes to the relaxant effect.

Caffeine has been shown to increase cyclic AMP content by inhibiting phosphodiesterase (Polson *et al.*, 1978; Fredholm *et al.*, 1979; Bray *et al.*, 1989). As cyclic AMP is known to cause relaxation of smooth muscle, we examined the effect of forskolin an activator of adenylate cyclase (Muller & Baer, 1983; Daly, 1984). A concentration of forskolin (3×10^{-7} M) that produces a concentration of cyclic AMP equivalent to that produced by 20 mM caffeine was used (Ozaki *et al.*, 1990). Caffeine completely relaxed strips precontracted with ACh or PDBu, but forskolin although able to relax ACh-induced contractions completely did not affect PDBu-induced contractions. These results contrast with those of Forder *et al.* (1985) who showed that 25 μM forskolin inhibits phorbol ester (12-O-tetradecanoylphorbol-13-acetate, TPA)-induced contractions. The difference between that finding and ours may result from the use of different phorbol esters. Our data suggest that elevation of cyclic AMP content may not be a prerequisite for caffeine-induced relaxation. It is likely, however, that under conditions in which cyclic AMP elevation does occur, this contributes to the caffeine-induced relaxation.

In conclusion, caffeine has multiple cellular effects on pig coronary artery and is a powerful inhibitor of calcium-dependent and calcium-independent contractions. Caffeine-induced relaxation is accompanied by a decrease in $[Ca^{2+}]_i$, hyperpolarization and an increase in cyclic AMP (Polson *et al.*, 1978; Fredholm *et al.*, 1979; Bray *et al.*, 1989). These mechanisms are not observed under all conditions and the present observations suggest that they may be secondary. Our results and those of Ozaki *et al.* (1990) suggest that caffeine could mainly act through an unknown mechanism involving direct inhibition of actin-myosin filaments. Since contractions can be produced independently of Ca^{2+} and myosin light chain phosphorylation (Singer & Baker, 1987; Hoar & Kerrick, 1988), and since, under certain conditions, caffeine is able to induce relaxation without affecting $[Ca^{2+}]_i$, it is clear that much remains to be learned of the mechanisms that control contraction and relaxation in smooth muscle.

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Kinetic studies on stereospecific recognition by the thromboxane A_2/prostaglandin H_2 receptor of the antagonist, S-145

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1 The mechanism for the stereospecific recognition of the antagonist S-145 by the thromboxane A_2 (TXA_2)/prostaglandin H_2 (PGH_2) receptor was examined by ligand-binding techniques in rat vascular smooth muscle cells (VSMCs) and in human platelet membranes.

2 Scatchard analysis revealed the existence of a single class of binding sites with the same maximum number for both [^{3}H]-(+)-S-145 and [^{3}H]-(−)-S-145 in both cell types. The dissociation constants (K_d) for the binding of the (+)-isomer in rat VSMCs and human platelet membranes were, respectively, 0.40 ± 0.03 and 0.20 ± 0.02 nM, each value being lower than that for the (−)-isomer (3.57 ± 0.74 and 2.87 ± 0.08 nM, respectively).

3 The rank orders of potency (K_i) for a series of TXA_2/PGH_2 ligands at inhibiting [^{3}H]-(+)-S-145 binding were highly correlated with those determined for [^{3}H]-(−)-S-145 binding in both cell preparations.

4 Kinetic analysis of the binding of both radioligands revealed a much lower dissociation rate constant (k_{-1}) and a slightly greater association rate constant (k_1) for the (+)-isomer compared to those for the (−)-isomer.

5 These results suggest that it is at the stage of dissociation from the TXA_2/PGH_2 receptor that the stereochemistry of the optical isomers of S-145 confers their difference in affinity for these receptors in rat VSMCs and human platelet membranes.

Keywords: Thromboxane A_2; thromboxane A_2/prostaglandin H_2 receptor; receptor antagonist; S-145; stereospecificity; rat aortic vascular smooth muscle cells; binding kinetics.

Introduction

Thromboxane A_2 (TXA_2), a metabolite of arachidonic acid, is potent both as an inducer of platelet aggregation and as a vasoconstrictor agent (Hamberg, 1975; Svensson *et al.*, 1977). It is thought to be responsible for the pathogenesis of some cardiovascular and thromboembolic disorders (Halushka & Lefer, 1987; Ogletree, 1987). (±)-S-145, a potent and selective TXA_2/prostaglandin H_2 (PGH_2) receptor antagonist (Narisada *et al.*, 1988), has been successfully used to characterize the TXA_2/PGH_2 receptors in platelets (Hanasaki & Arita, 1988a; Hanasaki *et al.*, 1989a), vascular smooth muscle cells (VSMCs) (Hanasaki *et al.*, 1988a; 1989b) and vascular endothelial cells (Hanasaki *et al.*, 1988b). It has two stereoisomers, and (+)-S-145 has been shown to possess a greater affinity for the TXA_2/PGH_2 receptors than the (−)-isomer (Hanasaki & Arita, 1988b). In addition, (+)-S-145 was observed to be a longer lasting antagonist of some TXA_2-mediated pharmacological responses both *in vivo* (Hori *et al.*, 1989) and *in vitro* (Nakajima & Ueda, 1989; Hanasaki *et al.*, 1989b). Similar stereospecific recognition of optical isomers has been reported for various types of receptors (Burgisser *et al.*, 1981); however, little is known about the underlying mechanism.

In the present study, we examined the differences in the binding mode between [^{3}H]-(+)-S-145 and [^{3}H]-(−)-S-145 in rat VSMCs and human platelet membranes. This is the first report of a kinetic analysis of the stereospecific recognition by TXA_2/PGH_2 receptors of the optical isomers of S-145.

Methods

Chemical preparation of [^{3}H]-S-145

(−)-(1S, 2R, 3R, 4R)-(5Z)-7-(3-[4-^{3}H]-phenyl sulphonylamino-bicyclo[2.2.1]hept-2-yl)hept-5-enoic acid sodium salt, [^{3}H]-(−)-S-145 sodium salt, was prepared as follows. To a stirred solution of 40 mg (0.18 mmol) methyl (−)-(1S, 2R, 3R, 4R)-(5Z)-7-(3-aminobicyclo[2.2.1]hept-2-yl)hept-5-enoate (Narisada *et al.*, 1988) in anhydrous benzene (2 ml) were added 20 mg (0.2 mmol) triethylamine and a solution of [4-^{3}H]-phenylsulphonyl chloride (100 mCi, 23.7 Ci mmol^{-1}, Amersham) in anhydrous benzene (11.5 ml). The mixture was concentrated *in vacuo* (60 mmHg) at 35°C to about 2 ml and stirred for 2.5 h at room temperature. The reaction mixture was evaporated *in vacuo* below 25°C, leaving a viscous oil which was purified by column chromatography on silica gel (Merck No. 7734, 900 mg; elution with benzene-ethylacetate 9:1), giving pure methyl (−)-(1S, 2R, 3R, 4R)-(5Z)-7-(3-[4-^{3}H]-phenylsulphonylaminobicyclo [2.2.1]- hept-2-yl)hept-5-enoate ([^{3}H]-(−)-S-145 methyl ester) (80 mCi, 0.0033 mmol) as a viscous oil. [^{3}H]-(−)-S-145 methyl ester was converted into [^{3}H]-(−)-S-145 sodium salt by saponification with 1 N sodium hydroxide (0.1 ml) in methanol (0.8 ml) by means of stirring for 15 h at room temperature. The mixture was then evaporated *in vacuo* below 25°C, giving crude [^{3}H]-(−)-S-145 sodium salt as a solid residue which was purified by chromatography (Waters SEP-PAK$_{c\text{-}18}$; elution with ethanol-water 1:4). The fractions containing the compound required in almost pure form were combined and evaporated *in vacuo* to dryness, giving [^{3}H]-(−)-S-145 sodium salt (71.5 mCi, 1.2 mg, 24.07 Ci mmol^{-1}, radiochemical purity 99.9%) in a 71.5% yield based on [4-^{3}H]-phenylsulphonyl chloride. The sodium salt of [^{3}H]-(−)-S-145 was dissolved in 95% ethanol, and the radioactive concentration was adjusted to 1.0 mCi ml^{-1}. Radiochemical purity was measured by thin layer chromatography (t.l.c.) autoradiography followed by liquid scintillation counting and high performance liquid chromatography (h.p.l.c.) (Nucleosil 5C$_{18}$, 4.6 mm × 15 cm; mobile phase, CH_3CN:CH_3OH:H_2O:CH_3COOH = 300:200:300:1; detection, u.v. 220 nm and radioactivity measured with a Packard Trace II-7150 detector). The intermediate and product were identified with authentic unlabelled compounds by comparison of t.l.c. (R_F) and h.p.l.c. (retention time). The R_F value of

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(−)-S-145 was 0.31 with chloroform/methanol (95/5, v/v) as the solvent system, and the retention time obtained by h.p.l.c. analysis was 7.3 min at the flow rate of 1.5 ml min^{-1}.

(+)-(1R, 2S, 3S, 4S)-(5Z)-7-(3-[4-^{3}H]-phenylsulphonylaminobicyclo[2.2.1]hept-2-yl)hept-5-enoic acid sodium salt, [^{3}H]-(+)-S-145 sodium salt (31.9 mCi, 0.48 mg, 26.4 Ci $mmol^{-1}$, radiochemical purity 99.9%) was prepared from 27 mg (0.114 mmol) methyl (+)-(1R, 2S, 3S, 4S)-(5Z)-7-(3-aminobicyclo[2.2.1]hept-2-yl)hept-5-enoate (Narisada *et al.*, 1988) and [4-^{3}H]-phenylsulphonylchloride (99 mCi, 29 Ci $mmol^{-1}$, Amersham) by an essentially similar method to that used for the synthesis of [^{3}H]-(−)-S-145 sodium salt.

Preparation of cultured vascular smooth muscle cells of rat aorta and human platelet membranes

Cultured rat aortic VSMCs were prepared as described previously (Hanasaki *et al.*, 1988a). Briefly, the cells were isolated from the medial explants of the aorta of 7-week-old male Sprague-Dawley rats by the method of Ross (1971). They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% foetal calf serum (Gibco, Grand Island, NY), penicillin (100 units ml^{-1}) and streptomycin (100 $\mu g\,ml^{-1}$) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The preparation of human platelet membranes was carried out as described by Hanasaki *et al.* (1989a). In brief, human blood, drawn by venepuncture from healthy male volunteers, was placed in 0.15 vol. of acid citrate dextrose (85 mM trisodium citrate, 70 mM citric acid and 110 mM glucose) containing 12 $\mu g\,ml^{-1}$ PGE_1. Platelet-rich plasma (PRP) was obtained by centrifugation at 160 *g* for 10 min and the platelets were then resuspended in ice-cold lysing buffer (5 mM Tris-HCl, pH 7.4, containing 5 mM EDTA, 10 μM indomethacin and 0.3 mM PMSF). After homogenization followed by centrifugation at 30,000 *g* for 10 min at 4°C, the pellets were suspended in incubation buffer (50 mM Tris-HCl, pH 7.4, containing 5 mM EDTA, 10 μM indomethacin and 0.3 mM PMSF) to give a protein concentration of 2 mg ml^{-1} and stored at −80°C until use. Protein concentration was determined by the method of Lowry *et al.* (1951) with human serum albumin as a standard.

Binding assays

For the binding assays with rat VSMCs, confluent VSMCs in 35 mm diameter dishes (approximately 5 × 10^5 cells/dish) were washed twice with phosphate-buffered saline. The incubations were carried out at 25°C in culture dishes in a total volume of 1 ml binding medium (Hanks' solution, pH 7.6, containing 0.1% BSA) with each tritium-labelled TXA_2/PGH_2 receptor ligand at the indicated concentration. After the reaction was stopped by rapid removal of the medium, the cells were washed three times with ice-cold 0.9% NaCl, and then solubilized in 1 N NaOH. The content of radioactivity was analyzed after adding an equal volume of 1 N HCl. The specific binding is defined as the difference between binding in the presence and absence of the unlabelled antagonist (10 μM). The specific binding of 2.0 nM [^{3}H]-(+)-S-145 and 10.5 nM [^{3}H]-(−)-S-145, respectively, represented 90 and 70% of the total binding. The binding assays in human platelet membranes were carried out according to a method described previously (Hanasaki *et al.*, 1989a). Briefly, platelet membranes (80 μg) were incubated with each tritium-labelled TXA_2/PGH_2 receptor ligand in a total volume of 0.5 ml at 25°C. After the incubation, ice-cold 0.9% NaCl was added to each tube, and the reaction mixture was immediately filtered by suction through a Whatman GF/C glass filter. The filter was then washed three times with ice-cold 0.9% NaCl, and analyzed for radioactivity. The specific binding of 3.6 nM [^{3}H]-(+)-S-145 and 11.2 nM [^{3}H]-(−)-S-145, respectively, represented 96 and 92% of the total binding.

Materials

U46619, PGE_1, PGE_2, PGD_2, $PGF_{2\alpha}$ and bovine serum albumin (BSA) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). TXB_2 was purchased from Funakoshi (Osaka, Japan). Phenylmethylsulphonyl fluoride (PMSF) and ethylenediamine tetraacetic acid (EDTA) were from Nakarai Chemicals (Kyoto, Japan). (±)-S-145, and its (+)- and (−)-isomers, were synthesized at Shionogi Research Laboratories, Osaka (Narisada *et al.*, 1988). ONO3708 ((9,11),(11,12)-dideoxa-9α, 11α-dimethylmethano-11, 12-methano-13, 14-dihydro - 12 - aza - 14 - oxo - 15 - cyclopentyl - 16,17,18,19,20 - pentanol-15-epi-TXA_2) was prepared as described previously (Hanasaki *et al.*, 1989a). Other chemicals were of analytical grade and were obtained from commercial sources.

Statistical analysis

Linear regression analysis of the binding data was performed according to standard methods (Nelder & Mead, 1965).

Results

Equilibrium binding studies of [^{3}H]-(+)-S-145 and [^{3}H]-(−)-S-145

The characteristics of the equilibrium binding of [^{3}H]-(+)-S-145 and [^{3}H]-(−)-S-145 were first examined. Saturation

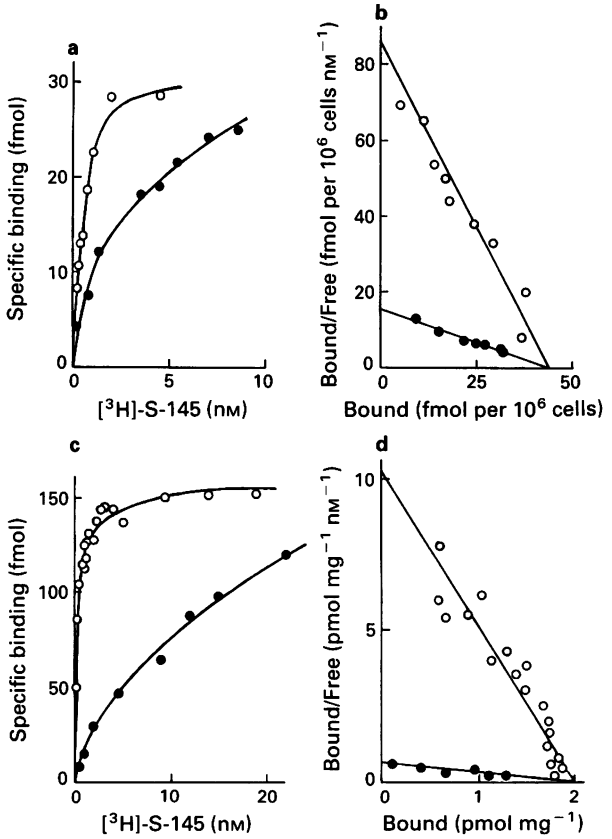

Figure 1 Binding of [^{3}H]-S-145 to rat vascular smooth muscle cells (VSMCs) and human platelet membranes. Saturation curves for the equilibrium binding of [^{3}H]-(+)-S-145 (○) or [^{3}H]-(−)-S-145 (●) to rat VSMCs (a) and human platelet membranes (c) are shown. Rat VSMCs were incubated with increasing concentrations of [^{3}H]-(+)-S-145 or [^{3}H]-(−)-S-145 at 25°C for 90 and 60 min, respectively. Human platelet membranes were incubated with increasing concentrations of [^{3}H]-(+)-S-145 or [^{3}H]-(−)-S-145 at 25°C for 40 and 30 min, respectively. Non-specific binding in the presence of 10 μM unlabelled ligand was subtracted from each point, which is representative of duplicate determinations in three experiments; (b) and (d) are the Scatchard plots of the same data as in (a) and (c), respectively, showing the lines obtained by linear regression.

Table 1 Comparison of K_d and B_{max} values for binding of the isomers of S-145 to rat vascular smooth muscle cells (VSMCs) and human platelet membranes

Ligand	K_d (nM)	B_{max} (fmol)
	Rat VSMCs	
[^{3}H]-(+)-S-145	0.40 ± 0.03	48.7 ± 8.9 per 10^6 cells
[^{3}H]-(−)-S-145	3.57 ± 0.74	44.8 ± 1.7
	Human platelet membranes	
[^{3}H]-(+)-S-145	0.20 ± 0.02	1970 ± 36 mg^{-1} protein
[^{3}H]-(−)-S-145	2.87 ± 0.08	1880 ± 82

Values were obtained by Scatchard analysis. Data are means ± s.e.mean of 3 experiments.

studies of the binding to rat VSMCs and human platelet membranes revealed that non-specific binding increased linearly with rising concentrations of each ligand, whereas the specific binding was saturable in both cell preparations (Figure 1a,c). Scatchard analysis of these data indicated the existence of a single class of binding sites for each optical isomer of S-145 in rat VSMCs as well as human platelet membranes (Figure 1b,d). The K_d value for the [^{3}H]-(+)-isomer was almost 10 times smaller than that of the [^{3}H]-(−)-isomer (Table 1). The maximum number of binding sites (B_{max}) was found to be almost the same for the optical isomers of S-145 in each preparation.

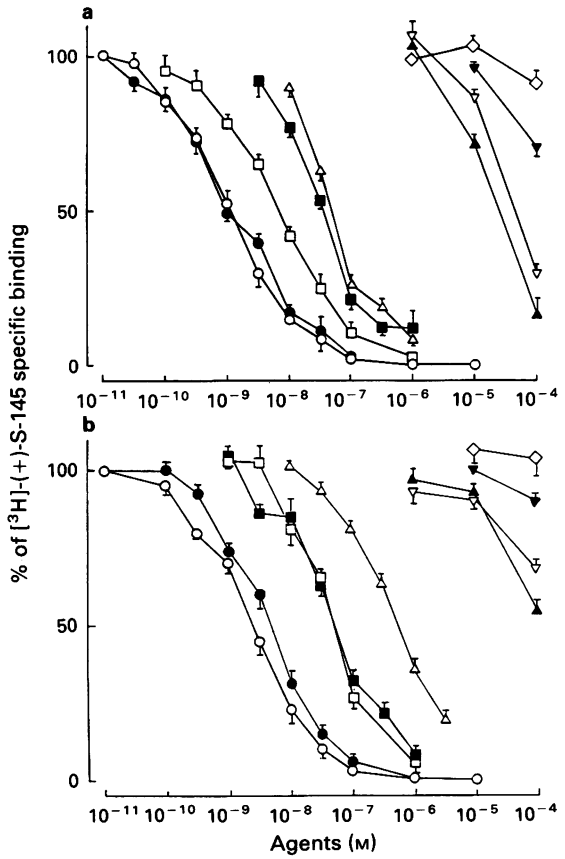

Figure 2 Inhibition of specific [^{3}H]-(+)-S-145 binding to rat vascular smooth muscle cells (VSMCs) (a) and human platelet membranes (b) by S-145 and related compounds. Rat VSMCs were incubated with 2.0 nM [^{3}H]-(+)-S-145 in the presence of various concentrations of (+)-S-145 (○), (±)-S-145 (●), (−)-S-145 (□), ONO3708 (■), U46619 (△), prostaglandin D_2 (PGD_2) (▲), $PGF_{2\alpha}$ (▽), PGE_2 (▼) and TXB_2 (◇). Incubation was carried out at 25°C for 90 min. Human platelet membranes were incubated with 1.8 nM [^{3}H]-(+)-S-145 at 25°C for 40 min in the presence of the same compounds. The control value (100%) was defined as the specific binding of [^{3}H]-(+)-S-145 in the absence of these compounds. Each point is mean of three independent experiments performed in triplicate; vertical bars show s.e.mean.

Table 2 Inhibition of [^{3}H]-(+)-S-145 and [^{3}H]-(−)-S-145 binding to rat vascular smooth muscle cells (VSMCs) and human platelet membranes by thromboxane A_2 (TXA_2)/prostaglandin H_2 (PGH_2) receptor ligands

	K_i (nM)	
Ligand	[^{3}H]-(+)-*S-145*	[^{3}H]-(−)-*S-145*
	Rat VSMCs	
(+)-S-145	0.23 ± 0.08	0.32 ± 0.06
(±)-S-145	0.29 ± 0.07	0.41 ± 0.02
(−)-S-145	1.7 ± 0.3	1.7 ± 0.1
ONO3708	7.3 ± 0.3	7.9 ± 1.5
U46619	11.3 ± 1.6	20.7 ± 5.0
	Human platelet membranes	
(+)-S-145	0.25 ± 0.02	0.20 ± 0.06
(±)-S-145	0.45 ± 0.03	0.41 ± 0.14
(−)-S-145	4.7 ± 0.2	3.2 ± 1.0
ONO3708	5.0 ± 0.3	3.9 ± 1.2
U46619	52.7 ± 1.4	49.8 ± 9.7

The K_i values were determined according to the equations of Cheng & Prusoff (1973), using the IC_{50} values derived from the inhibition data (Figure 2) and the K_d values obtained by Scatchard analysis (Figure 1). Data are means ± s.e.mean of 3 experiments. The correlation coefficients between the negative logarithms of the K_i values of the five TXA_2/PGH_2 receptor ligands against [^{3}H]-(+)-S-145 binding and against [^{3}H]-(−)-S-145 binding were 0.99 in both rat VSMCs and human platelet membranes.

Binding specificity of [^{3}H]-(+)-S-145 and [^{3}H]-(−)-S-145 in rat vascular smooth muscle cells and human platelet membranes

Several structurally dissimilar compounds were examined for their abilities to compete with the specific binding of [^{3}H]-(+)-S-145 or [^{3}H]-(−)-S-145. Five TXA_2/PGH_2 receptor ligands inhibited the binding of both [^{3}H]-(+)-S-145 (Figure 2a) and [^{3}H]-(−)-S-145 (data not shown) to rat VSMCs in a concentration-dependent manner, and the potency series, as given by the IC_{50} values for unlabelled S-145, was found to be (+)-isomer > racemate > (−)-isomer. U46619, a stable TXA_2-mimetic agonist (Coleman *et al.*, 1980), also completely inhibited binding of both ligands, whereas PGD_2, PGE_2, $PGF_{2\alpha}$ and TXB_2 were inhibitory only at concentrations greater than 10 μM, demonstrating the binding selectivity of both optical isomers for TXA_2/PGH_2 receptors. Similar displacement data were obtained with human platelet membranes (Figure 2b). The IC_{50} values for the respective ligands in both cell preparations were obtained from the displacement data, and then the K_i values, given in Table 2, were calculated by the Cheng-Prusoff equation (Cheng & Prusoff, 1973), using the respective K_d values shown in Table 1. The negative logarithms of the K_i values of the five TXA_2/PGH_2 receptor ligands against [^{3}H]-(+)-S-145 binding were highly correlated with the corresponding K_i values for inhibition of [^{3}H]-(−)-S-145 binding in both rat VSMCs (r = 0.99) and human platelet membranes (r = 0.99).

Kinetic analysis of the binding of [^{3}H]-(+)-S-145 and [^{3}H]-(−)-S-145 to rat vascular smooth muscle cells and human platelet membranes

In order to characterize further the binding of [^{3}H]-(+)-S-145 and [^{3}H]-(−)-S-145, kinetic analysis was performed with rat VSMCs and human platelet membranes. As shown in Figure 3a, the specific binding of 1.9 nM [^{3}H]-(+)-isomer and 10.4 nM [^{3}H]-(−)-isomer to rat VSMCs followed similar time-

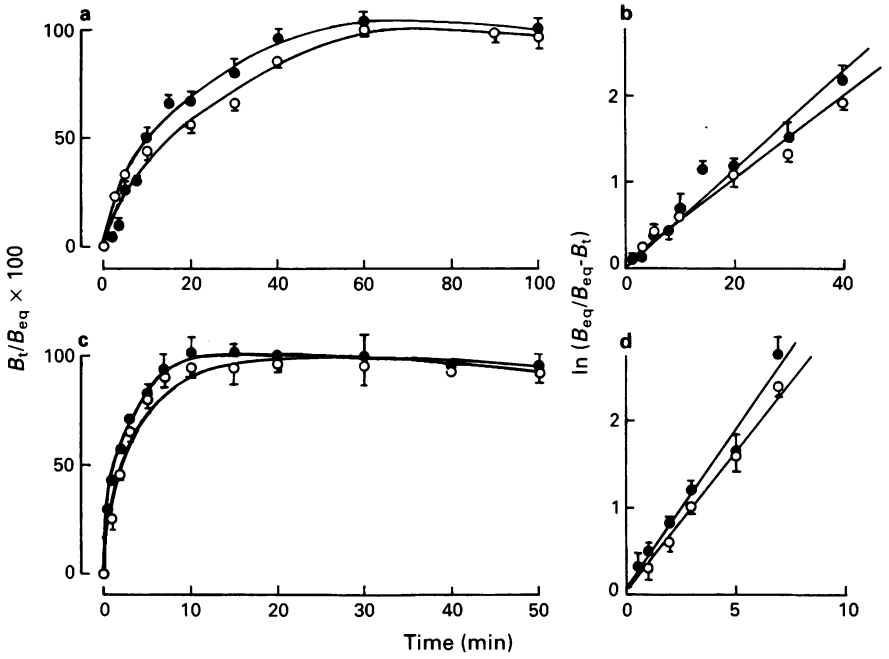

Figure 3 The time course of association of [^{3}H]-S-145 to rat vascular smooth muscle cells (VSMCs) (a) and human platelet membranes (c). Rat VSMCs were incubated with 1.9 nM [^{3}H]-(+)-S-145 (○) or 10.4 nM [^{3}H]-(−)-S-145 (●) at 25°C for the indicated times. Human platelet membranes were incubated with 3.5 nM [^{3}H]-(+)-S-145 or 11.0 nM [^{3}H]-(−)-S-145 at 25°C. The specific binding is expressed as a percentage of the specific binding at equilibrium. B_{eq} is the specific binding at equilibrium and B_t is the amount of [^{3}H]-S-145 specifically bound at any indicated time. Each point is the mean of three independent experiments; vertical bars show s.e.mean; (b) and (d) are the pseudo first-order rate plots of the data taken from (a) and (c), respectively; the slope of this plot is k_{obs}.

dependent processes and reached equilibrium at 65 and 50 min, respectively. The observed rate constants (k_{obs}), derived from the slope of the pseudo first-order rate plot of these data (Figure 3b), were found to be $0.040 \pm 0.006\,min^{-1}$ for the [^{3}H]-(+)-isomer and $0.061 \pm 0.005\,min^{-1}$ for the [^{3}H]-(−)-isomer. Similar association experiments were performed with human platelet membranes and the k_{obs} values were calculated to be $0.24 \pm 0.06\,min^{-1}$ for 3.5 nM [^{3}H]-(+)-S-145 and $0.47 \pm 0.01\,min^{-1}$ for 11.0 nM [^{3}H]-(−)-S-145 (Figure 3c,d). Next, to determine the dissociation rate constant

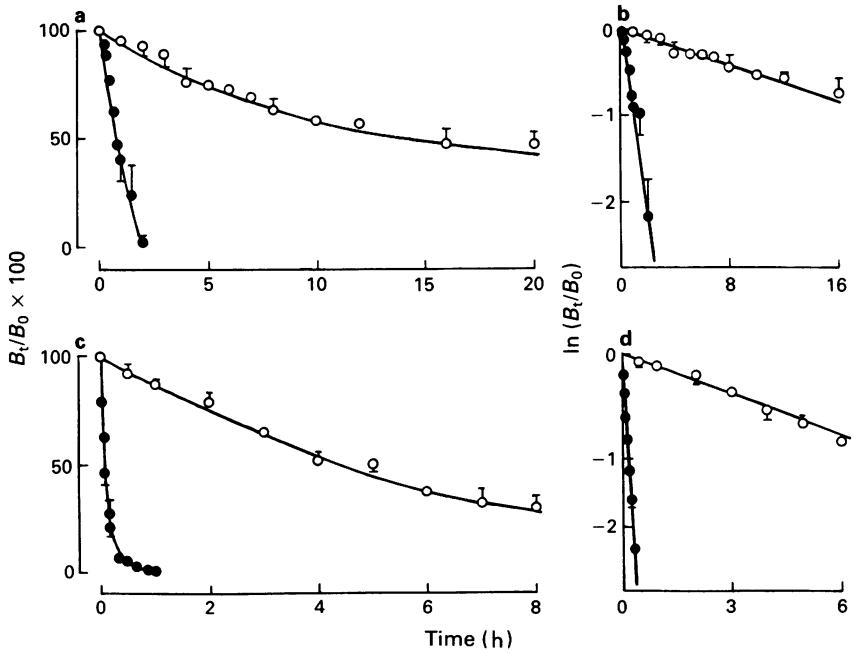

Figure 4 The time course of dissociation of [^{3}H]-S-145 from rat vascular smooth muscle cells (VSMCs) (a) and human platelet membranes (c). The association between rat VSMCs and 1.9 nM [^{3}H]-(+)-S-145 (○) or 10.4 nM [^{3}H]-(−)-S-145 (●) was carried out at 25°C for 90 and 60 min, respectively, and that of human platelet membranes with 3.5 nM [^{3}H]-(+)-S-145 or 11.0 nM [^{3}H]-(−)-S-145 was carried out at 25°C for 40 and 30 min, respectively. Next, 10 μM unlabelled ligand was added at time zero to initiate displacement of the ^{3}H-labelled antagonist from its specific binding site. The data are expressed with the value of the specific binding at time zero as 100%. B_t is the amount of [^{3}H]-S-145 specifically bound at any particular time, t, and B_0 is the amount at time zero. Each point is the mean of three independent experiments; vertical bars show s.e.mean; (b) and (d) are the linear transformation of the data taken from (a) and (c), respectively; the slope of the plot is k_{-1}.

Table 3 Kinetic, and derived equilibrium, constants for the binding of ^{3}H-labelled (+)-S-145 and (−)-S-145 to rat vascular smooth muscle cells (VSMCs) and human platelet membranes

Ligand	k_1 ($M^{-1}min^{-1}$)	k_{-1} (min^{-1})	$K_d = k_{-1}/k_1$ (nM)
	Rat VSMCs		
[^{3}H]-(+)-S-145	$2.4 \pm 0.4 \times 10^7$	0.0010 ± 0.0003	0.039 ± 0.005
[^{3}H]-(−)-S-145	$4.8 \pm 0.3 \times 10^6$	0.015 ± 0.003	3.1 ± 0.5
	Human platelet membranes		
[^{3}H]-(+)-S-145	$6.7 \pm 0.9 \times 10^7$	0.0028 ± 0.0003	0.043 ± 0.004
[^{3}H]-(−)-S-145	$3.0 \pm 0.2 \times 10^7$	0.14 ± 0.01	4.5 ± 0.3

The results shown are means ± s.e.mean of 3 experiments obtained in triplicate determinations. Experiments were performed as described in the legends of Figure 3 and Figure 4.

(k_{-1}), both cell preparations were incubated with each ^{3}H-labelled antagonist to ensure that the equilibrium had been reached. At an arbitrary zero time point, an excess of the respective unlabelled isomer of S-145 (10 μM) was added, and the time course of displacement of each radioligand from its binding site was determined. As shown in Figure 4a, in rat VSMCs the dissociation of [^{3}H]-(+)-S-145 from its specific binding sites was much slower than that of [^{3}H]-(−)-S-145. The times at which one-half of its equilibrium level had been reached were 960 min for the (+)-isomer and 50 min for the (−)-isomer, and the times at which complete dissociation had been achieved were, respectively, 72 and 2.5 h (data not shown). Similar dissociation patterns of [^{3}H]-(+)-S-145 and [^{3}H]-(−)-S-145 were observed with human platelet membranes (Figure 4c). Linear transformation of these data (Figure 4b,d) gave a k_{-1} value for the binding of each radioligand, and we calculated the value for k_1 (association rate constant), according to the equation $k_1 = (k_{obs} - k_{-1})/[L]$ (where [L] is radioligand concentration), and also determined the K_d value given by $K_d = k_{-1}/k_1$, all of which are summarized in Table 3. An extremely slow k_{-1} as well as a slightly greater k_1 value for the (+)-isomer were observed for both cell preparations.

Discussion

The present study describes a kinetic analysis for the stereospecific recognition by TXA_2/PGH_2 receptors of the optical isomers of S-145. We used cultured VSMCs from rat thoracic aorta and human platelet membranes as *in vitro* models since these cell preparations possess intrinsic TXA_2/PGH_2 receptors which, when activated, lead to the corresponding pharmacological responses (Ogletree, 1987; Hanasaki *et al.*, 1989b). However, in this study, the binding experiments for rat VSMCs were performed with cells which were adherent to culture dishes, instead of the trypsin-harvested cells which had been used previously (Hanasaki *et al.*, 1989b), since the treatment with trypsin and chymotrypsin causes destruction of the TXA_2/PGH_2 receptor-mediated responses in platelets (Lukasiewicz *et al.*, 1989). Scatchard analysis proved that the receptor density determined by the present method was about 5 fold greater than the levels obtained by the previous methods (Hanasaki *et al.*, 1989b) and showed that this occurred without any significant changes in the ligand affinities, thus demonstrating similar tryptic proteolysis of the TXA_2/PGH_2 receptors in rat VSMCs.

Scatchard analysis, as well as the displacement data, clearly showed that the binding affinity of [^{3}H]-(+)-S-145 for TXA_2/PGH_2 receptors was much greater than that of the (−)-isomer, which is consistent with the pharmacological data in vascular smooth muscle as well as in platelets (Hanasaki *et al.*, 1989b). Similar radioligand binding studies on the differences in the binding affinity between stereoisomers have been performed with several receptor systems (Burgisser *et al.*, 1981). For the γ-aminobutyric acid (GABA) receptor, Drew *et al.* (1984) reported different classes of binding sites for [^{3}H]-(−)- and [^{3}H]-(±)-baclofen, a GABA agonist, in synaptic membranes of rat cerebellum, which might account for their discrepancies in pharmacological potencies. However, for the optical isomers of S-145, Scatchard analysis revealed that the maximum number of binding sites for the (+)-isomer was virtually identical to that for the (−)-isomer (Table 1), suggesting that both isomers interact with the same binding sites. This conclusion is further supported by the high correlation between the inhibitory potencies (K_i) of several TXA_2/PGH_2 receptor ligands when competing with the binding of [^{3}H]-(+)- and [^{3}H]-(−)-S-145 (Table 2). Thus, the disparities between the stereoisomers of S-145 appear to arise from differences in their binding modes to the same TXA_2/PGH_2 receptor molecule.

The kinetic analysis of the radioligand binding in both cell preparations (Figures 3 and 4) revealed that the (+)-isomer possesses a slightly greater k_1 value than the (−)-isomer, which was almost the same as those of other TXA_2/PGH_2 antagonists, such as ONO3708 and SQ29,548 (Hanasaki *et al.*, 1989a). On the other hand, the dissociation rate constant (k_{-1}) for the (+)-isomer was found to be much smaller than that for the (−)-isomer as well as for other receptor antagonists (Hanasaki *et al.*, 1989a), which is consistent with the long-lasting action of (+)-S-145 in the antagonism of some TXA_2-mediated pharmacological responses *in vivo* (Hori *et al.*, 1989). These findings suggest that these TXA_2/PGH_2 receptors would strictly recognize the differences between the optical isomers of S-145 at the stage of dissociation from the receptors rather than at the stage of accessibility to the receptors.

The very much smaller k_{-1} value for the (+)-isomer would make a large contribution towards it having a much lower K_d value ($K_d = k_{-1}/k_1$) than that for the (−)-isomer, which would reflect the higher affinity of the (+)-isomer for the TXA_2/PGH_2 receptors in both preparations. However, a wide divergence (approximately 10 fold) was observed between the K_d values derived from kinetic analysis and those from Scatchard analysis for (+)-S-145 binding in each preparation, whereas a good correlation was observed for (−)-S-145 binding. Such a great difference between both the K_d values was also reported in the binding of (±)-S-145 which acts as a partial agonist to evoke shape change without aggregation and secretion responses in human platelets (Hanasaki *et al.*, 1989a). Our preliminary experiments have shown that (+)-S-145 also possesses higher partial agonist potency (by about ten times) than (−)-S-145 in human platelets (unpublished data). These observations might suggest the possibility that the discrepancy between the K_d values determined from kinetic analysis and Scatchard analysis arise from some conformational change in the TXA_2/PGH_2 receptors after the (+)-S-145 has bound which results from its potent partial agonist activity. Recently, conformational analyses verified that (±)-S-145 has energetically stable conformers similar to the putative active form (hairpin-like structure) of TXA_2 or U46619 (Ezumi *et al.*, 1990). Such a stereochemical comparison would offer further information about the mechanism for the differences in the dissociation pattern of the optical isomers of S-145. Since the TXA_2/PGH_2 receptor has been purified from human platelets (Ushikubi *et al.*, 1989) and its amino acid sequence has recently been determined (Hirata *et al.*, 1991), studies on the structure of its binding domains should also help further our knowledge of the interaction of (+)-S-145 with the TXA_2/PGH_2 receptor at the molecular level.

In conclusion, this paper presents the characterization of the stereospecific recognition of the TXA_2/PGH_2 receptor for the optical isomers of S-145 by kinetic analysis. The absence

of differences in high affinity binding of (+)-S-145 for the receptors between platelets and VSMCs indicated that (+)-S-145 may serve as a useful ligand for analysis of the role of TXA_2/PGH_2 receptors in certain pathophysiological processes. In addition, (+)-S-145 can be expected to be a long-lasting TXA_2/PGH_2 receptor antagonist for clinical use owing to its extremely slow dissociation from the TXA_2/PGH_2 receptors.

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Characterization of a novel, hydrophilic dihydropyridine, NKY-722, as a Ca^{2+} antagonist in bovine cultured adrenal chromaffin cells

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1 To characterize NKY-722, a novel hydrophilic dihydropyridine derivative, as a Ca^{2+} antagonist, we examined its effects on $^{45}Ca^{2+}$ influx, intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$), and release of noradrenaline and adrenaline in bovine cultured adrenal chromaffin cells.

2 NKY-722 had little effect on basal $^{45}Ca^{2+}$ influx into the resting cells, but inhibited high K^+ (35.9 mM)-evoked $^{45}Ca^{2+}$ influx in a concentration-dependent manner with an IC_{50} value of 5.2 nM.

3 NKY-722 inhibited high K^+-evoked increases in $[Ca^{2+}]_i$ in a concentration-dependent manner without effect on the resting $[Ca^{2+}]_i$.

4 NKY-722 had little effect on basal release of noradrenaline and adrenaline but inhibited high K^+-evoked release of noradrenaline and adrenaline in a concentration-dependent manner with IC_{50} values of 5.0 nM and 4.8 nM, respectively.

5 Nicardipine, a prototype of NKY-722, also inhibited high K^+-evoked $^{45}Ca^{2+}$ influx and release of noradrenaline and adrenaline in a concentration-dependent manner: the IC_{50} value for high K^+-evoked $^{45}Ca^{2+}$ influx was 51 nM, and the values for high K^+-evoked release of noradrenaline and adrenaline were 52 nM and 50 nM, respectively.

6 These results show that NKY-722 is a hydrophilic Ca^{2+} antagonist ten times more potent than nicardipine.

Keywords: Adrenal chromaffin cells; Ca^{2+} antagonist; Ca^{2+} influx; intracellular free Ca^{2+} concentration; Ca^{2+} channel; catecholamine release; dihydropyridine

Introduction

Voltage-dependent Ca^{2+} channels play an important role in the cellular regulation of Ca^{2+} movement. Biophysical and pharmacological criteria define at least three types of voltage-dependent Ca^{2+} channels, designated L (long-lasting), N (neuronal) and T (transient) in excitable cells (Hofmann *et al.*, 1987). The best characterized of these is the L channel, the target for a number of drugs. The 1,4-dihydropyridines (DHPs), phenylalkylamines and benzothiazepines, represented by nifedipine, verapamil and diltiazem, respectively, are the best known groups of compounds. These classic Ca^{2+} antagonists are widely employed therapeutically in a number of cardiovascular disorders and experimentally as tools for characterization of voltage-dependent, L-type Ca^{2+} channels and their cloning (Tanabe *et al.*, 1987; Mikami *et al.*, 1989). However, because of their hydrophobic character, their routes of administration and hence, their usage seem to be limited. To overcome the problem of hydrophobicity, a highly hydrophilic compound has recently been synthesized using nicardipine, a DHP, as a prototype. This compound, 3-(4-allyl-1-piperazinyl)-2,2-dimethyl propyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate dihydrochloride (NKY-722), has been shown to produce a long-lasting decrease in systemic arterial blood pressure in conscious spontaneously hypertensive rats (Osumi *et al.*, 1988), to inhibit Ca^{2+}-induced contraction of dog isolated mesenteric arteries (Osumi *et al.*, 1988) and to increase coronary blood flow (Imagawa *et al.*, 1989). From these indirect studies and because NKY-722 is a derivative of a classic dihydropyridine Ca^{2+} antagonist nicardipine, NKY-722 is considered to be a Ca^{2+} antagonist. As it may be important to have a hydrophilic Ca^{2+} antagonist from clinical and experimental viewpoints, we attempted to define NKY-722 as a Ca^{2+} antagonist by examining its effects on $^{45}Ca^{2+}$ influx, intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$), and release of noradrenaline and adrenaline in bovine cultured adrenal chromaffin cells.

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Methods

Isolation and culture of bovine adrenal chromaffin cells

Bovine adrenal chromaffin cells were isolated, purified and cultured as described previously (Fenwick *et al.*, 1978; Waymire *et al.*, 1983; Lee *et al.*, 1990). In brief, chromaffin cells were isolated by the retrograde perfusion of bovine adrenal medullae with collagenase and deoxyribonuclease I (DNase I), and then purified from other types of cells by density gradient centrifugation on Percoll and by differential plating. To determine catecholamine release and $^{45}Ca^{2+}$ uptake, purified cells were plated as monolayers in poly-L-lysine-coated 24 well- and 6 well-cluster plates (Costar, Cambridge, MA, U.S.A.) at a density of 4×10^5 and 2×10^6 cells/well, respectively. For determination of $[Ca^{2+}]_i$, the cells were kept in suspension on 150 mm-diameter plastic dishes (Falcon 1058; Becton, Dickinson and Co., Cockeysville, MD, U.S.A.) at a density of $1.0–1.6 \times 10^7$ cells/dish. The culture medium used to maintain the purified cells was Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal calf serum, 10 mM HEPES, 20 mM $NaHCO_3$, antimitotics (5-fluoro-2-deoxyuridine, 10^{-5} M; uridine, 10^{-5} M; and cytosine arabinoside, 10^{-5} M) to inhibit fibroblast growth, and antibiotics (penicillin G, 100 units ml^{-1}; streptomycin, 100 ng ml^{-1}; and nystatin, 25 units ml^{-1}). Cultures were maintained at 37°C in a humid atmosphere gassed with 5% CO_2 in air, and experiments were performed after 3 or 4 days of culture.

Measurement of $^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ uptake into cultured chromaffin cells was determined as described recently (Lee *et al.*, 1990). The cells were washed three times with Ca^{2+}-free Krebs-HEPES buffer (composition

(mM): NaCl 140, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, glucose 11 and HEPES 15; adjusted to pH 7.4), and then preincubated in the same buffer at 37°C for 10 min. After preincubation, the cells were stimulated by incubating them in Ca^{2+}-free high K^+ (35.9 mM) Krebs-HEPES buffer (with an equimolar reduction in Na^{2+} concentration) containing 2 μCi of $^{45}CaCl_2$ (specific activity, 10–40 mCi mg^{-1} of calcium) at 37°C for various times (total $^{45}Ca^{2+}$ uptake). For determination of basal uptake of $^{45}Ca^{2+}$, the cells were incubated without stimulation in Ca^{2+}-free Krebs-HEPES buffer containing 2 μCi of $^{45}CaCl_2$. When the effects of Ca^{2+} antagonists were examined, the drugs were present during preincubation and incubation. At the end of each incubation period, the reaction was terminated by rapidly aspirating the $^{45}Ca^{2+}$-containing medium, and the cells were washed four times with 3 ml of ice-cold Ca^{2+}-free Krebs-HEPES buffer. After washing, the cells were solubilized in 1 ml of 10% Triton X-100, and the $^{45}Ca^{2+}$ taken up into the cells was determined in a Packard Tri-Carb 4000 liquid scintillation counter. In the text, the data are shown for only high K^+-evoked $^{45}Ca^{2+}$ uptake, which is the difference between total and basal uptake. $^{45}Ca^{2+}$ uptake was expressed in terms of the initial specific activity in the incubation medium.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined according to the method of Grynkiewicz *et al.* (1985) as modified by Lee *et al.* (1990) using a Ca^{2+} indicator fura-2. The cells, which had been kept in suspension, were washed twice with fura-2 loading buffer and collected by centrifugation. The composition of the fura-2 loading buffer was the same as that of Ca^{2+}-free Krebs-HEPES buffer except that it contained 1 mM Ca^{2+}. The cells were resuspended in the fura-2 loading buffer containing 3 μM fura-2 acetoxymethylester at a density of 2×10^6 cells ml^{-1} and incubated at 37°C for 30 min for loading of fura-2. After the incubation, the cells were washed with bovine serum albumin/fura-2 loading buffer (fura-2 loading buffer containing 5 mg ml^{-1} bovine serum albumin) to remove unloaded fura-2. The cells were resuspended in 2 ml of fresh fura-2 loading buffer and transferred to a quartz cuvette for measurement of fluorescence. Fluorescence measurements were made in a JASCO (Japan Spectroscopic Co., Ltd., Tokyo, Japan) Ca^{2+} analyzer (model CAF-100). Excitation wavelengths were set at 340 nm and 380 nm and an emission wavelength at 510 nm (Grynkiewicz *et al.*, 1985). $[Ca^{2+}]_i$ values were calculated from fura-2 fluorescence ratios (R) according to the following equation:

$$[Ca^{2+}]_i = K(R - R_{min})/(R_{max} - R)$$

where R_{min} and R_{max} are the ratios, e.g., 340 nm/380 nm, obtained at zero and saturating Ca^{2+} concentrations and K is the product K_D(Fo/Fs), where K_D is the effective dissociation constant (224 nM), Fo is the 380 nm excitation signal in the absence of Ca^{2+}, and Fs is the 380 nm excitation signal at the saturating Ca^{2+} concentration (Grynkiewicz *et al.*, 1985). For determination of the saturating Ca^{2+} concentration, digitonin, a detergent, was added to the incubation medium at a final concentration of 20 μM. For determination of the zero Ca^{2+} concentration, EGTA, a Ca^{2+} chelator together with 100 μl of 1 M Tris-HCl (pH 8.6), was added to the incubation medium at a final concentration of 5 mM after treatment with digitonin.

Determination of catecholamine release

When catecholamine release from cultured chromaffin cells was measured, the culture medium was discarded, and the cells were washed three times with fresh Krebs-HEPES buffer (composition mM: NaCl 140, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.2, glucose 11 and HEPES 15; adjusted to pH 7.4). After the cells were preincubated in Krebs-HEPES buffer at 37°C for 10 min, the cells were stimulated by incubating them in 1 ml of high K^+ Krebs-HEPES buffer at 37°C for 6 min, the minimum time required for catecholamine release to reach a plateau (Lee *et al.*, 1990). The reaction was terminated by rapidly removing the incubation medium which was transferred to another set of test tubes containing 1 ml of 0.2 M perchloric acid. For extraction of catecholamine remaining in the cells, the cells were lysed by addition of 1 ml 0.4 M perchloric acid to each well, and scraped off. When the effects of Ca^{2+} antagonists were examined, the drugs were present during preincubation and incubation. Catecholamines, which were released into the medium and remained in the cells, were quantified by high performance liquid chromatography (h.p.l.c.). (Hitachi LC 655; Hitachi Co., Ltd., Tokyo, Japan) with electrochemical detection as described in detail elsewhere (Miwa *et al.*, 1986; Lee *et al.*, 1987). Results were expressed as percentage release of the total cellular catecholamine content.

Drugs

Chemicals were obtained from the following sources: $^{45}CaCl_2$, from Amersham, Arlington Heights, IL, U.S.A.; DNase I, 5-fluoro-2-deoxy-uridine and poly-L-lysine bromide, from Sigma, St. Louis, MO, U.S.A.; penicillin G and streptomycin, from Meiji Seika, Tokyo, Japan; cytosine arabinoside, from Kohjin, Tokyo, Japan; Percoll, from Pharmacia, Uppsala, Sweden; foetal calf serum, nystatin suspension and Dulbecco's modified Eagle's medium, from GIBCO, Grand Island, NY, U.S.A.; fura-2 acetoxymethyl ester, HEPES and EGTA, from Dohjin Chemical Institute, Kumamoto, Japan; bovine serum albumin, collagenase, uridine, digitonin and Triton X-100, from Wako Pure Chemical Industries, Osaka, Japan. NKY-722 was a generous gift from Kyoto Pharmaceutical Industries, Kyoto, Japan.

Statistical analysis

All results are expressed as means ± s.e.mean. The data were subjected to a two-way analysis of variance, and when significant F values were encountered, Newman-Keuls' multiple-range test was used to test for significant differences between treatment means (Steel & Torrie, 1960). A probability level of $P < 0.05$ was considered to be statistically significant.

To estimate the IC_{50} values for inhibition of high K^+-evoked catecholamine release and $^{45}Ca^{2+}$ uptake, sigmoid concentration-response curves were converted into straight lines by plotting values on the ordinate scale as log (Y/100 − Y): where Y is 100 × the ratio (the response in the presence of a given concentration of Ca^{2+} antagonist/the response in the absence of Ca^{2+} antagonist). The intercept with the abscissa scale (Y = 0) gives the IC_{50} values.

Results and Discussion

Figure 1 shows the time course of high K^+-evoked $^{45}Ca^{2+}$ uptake into cultured adrenal chromaffin cells in the absence or presence of NKY-722. NKY-722 had little effect on basal Ca^{2+} uptake into the resting cells (data not shown). When the cells were stimulated by high K^+ in the absence of NKY-722, high K^+-evoked $^{45}Ca^{2+}$ uptake into the cells increased rapidly and reached a plateau within 4 min. In the presence of 10^{-8} M NKY-722, the rate of the $^{45}Ca^{2+}$ uptake was markedly reduced but 20 min after addition of $^{45}Ca^{2+}$ to the medium, the amount of $^{45}Ca^{2+}$ taken up into the cells reached the same level as in the absence of NKY-722. In the presence of a higher concentration of NKY-722 (10^{-6} M), the $^{45}Ca^{2+}$ uptake was completely inhibited for up to 8 min.

It is generally accepted that the slope of the early increasing phase of $^{45}Ca^{2+}$ uptake curve is considered to represent the rate of Ca^{2+} influx through Ca^{2+} channels (Borle 1981; Artalejo *et al.*, 1987). In contrast, the late plateau phase is considered to represent the size of the intracellular exchangeable Ca^{2+} pool (Borle 1981; Artalejo *et al.*, 1987). On the other hand, in the adrenal chromaffin cells, depolarization of the

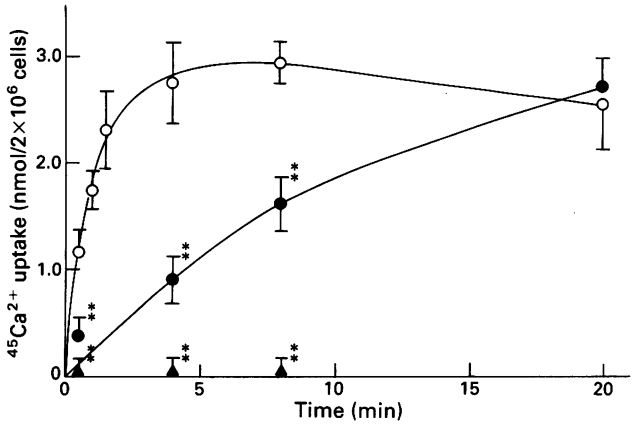

Figure 1 Time course of high K^+-evoked $^{45}Ca^{2+}$ uptake into cultured adrenal chromaffin cells in the absence or presence of NKY-722. After preincubation in Ca^{2+}-free Krebs-HEPES medium at 37°C for 10 min, the cells were stimulated by incubating them in high K^+ medium containing 2 μCi of $^{45}CaCl_2$ at 37°C for various times and the $^{45}Ca^{2+}$ taken up into the cells was determined (total uptake). Basal $^{45}Ca^{2+}$ uptake (data not shown) was determined by incubating cells in normal K^+ medium. When the effects of NKY-722 on $^{45}Ca^{2+}$ uptake were examined, NKY-722 was added to both preincubation and incubation medium. The data shown are high K^+-evoked $^{45}Ca^{2+}$ uptake, which was determined by subtracting basal uptake from total uptake: (○) high K^+-evoked $^{45}Ca^{2+}$ uptake in the absence of NKY-722; (●) and (▲) high K^+-evoked $^{45}Ca^{2+}$ uptake in the presence of 10^{-8} M and 10^{-6} M NKY-722, respectively. Data are means of three separate experiments, each done in triplicate; vertical bars show s.e.mean. ** $P < 0.01$; significantly different from the control value obtained in the absence of NKY-722.

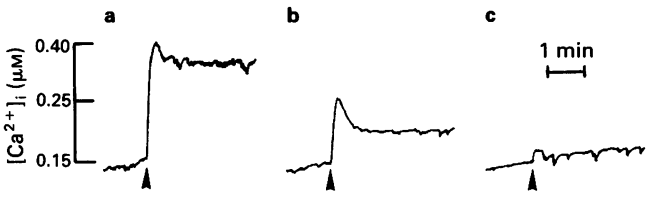

Figure 3 Typical tracings illustrating the effects of NKY-722 on high K^+-evoked increases in $[Ca^{2+}]_i$ in suspended adrenal chromaffin cells. Suspended cells were loaded with fura-2 and transferred to a quartz cuvette for measurement of the fluorescence. Ten minutes after addition of 50 μl of vehicle or NKY-722 solution, the cells were stimulated by addition of 40 μl 1.8 M KCl (final concentration, 35.9 mM) to the cuvette as indicated by arrowheads in the absence (a) or presence of NKY-722 (b, 10^{-8} M; c, 10^{-6} M).

plasma membrane by high K^+ stimulation is known to activate voltage-dependent, L-type Ca^{2+} channels in the membrane (Cena *et al.*, 1983; Vaghy *et al.*, 1987). Thus the present results suggest that NKY-722 inhibits Ca^{2+} influx through voltage-dependent, L-type Ca^{2+} channels but it does not affect the size of the intracellular exchangeable Ca^{2+} pool.

Figure 2 shows the effect of various concentrations of NKY-722 or nicardipine on high K^+-evoked $^{45}Ca^{2+}$ uptake into cultured adrenal chromaffin cells. Basal $^{45}Ca^{2+}$ uptake was virtually unaffected by NKY-722 or nicardipine (data not shown). High K^+-evoked $^{45}Ca^{2+}$ uptake was inhibited by NKY-722 or nicardipine in a concentration-dependent manner. The IC_{50} values for inhibition of high K^+-evoked $^{45}Ca^{2+}$ uptake were 5.2 ± 0.2 nM ($n = 9$) for NKY-722 and 51 ± 2 nM ($n = 9$) for nicardipine. Thus NKY-722 seems to be ten times more potent than its prototype nicardipine as a Ca^{2+} antagonist.

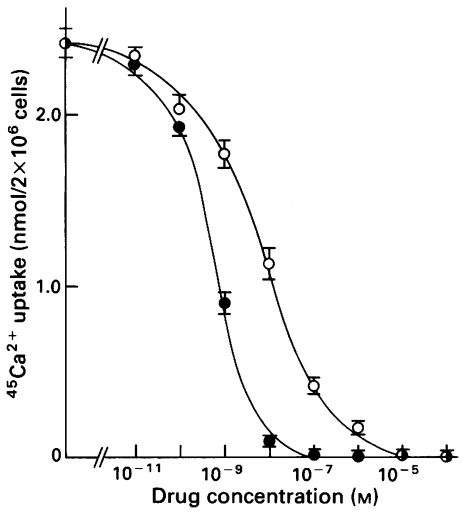

Figure 2 Effects of various concentrations of NKY-722 or nicardipine on high K^+-evoked $^{45}Ca^{2+}$ uptake into cultured adrenal chromaffin cells. After preincubation in Ca^{2+}-free Krebs-HEPES medium for 10 min at 37°C, the cells were stimulated by incubating them in high K^+ medium containing 2 μCi of $^{45}CaCl_2$ at 37°C for 1 min and the $^{45}Ca^{2+}$ taken up into the cells was determined (total uptake). Basal $^{45}Ca^{2+}$ uptake (data not shown) was determined by incubating cells in normal K^+ medium. When the effects of Ca^{2+} antagonists on $^{45}Ca^{2+}$ uptake were examined, Ca^{2+} antagonists were added to both preincubation and incubation medium. The data shown are high K^+-evoked $^{45}Ca^{2+}$ uptake, which was determined by subtracting basal uptake from total uptake: (●) NKY-722; (○) nicardipine; (◑) represents both NKY-722 and nicardipine. Data are means of three separate experiments, each done in triplicate; vertical bars show s.e.mean.

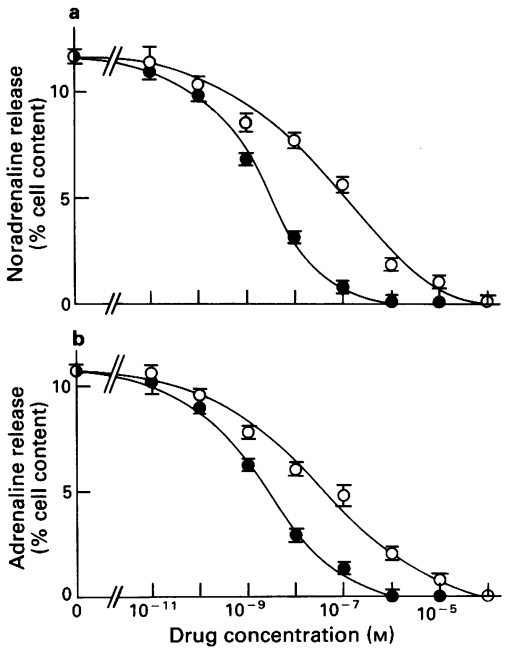

Figure 4 Effects of various concentrations of NKY-722 or nicardipine on high K^+-evoked release of noradrenaline (a) and adrenaline (b) from cultured adrenal chromaffin cells. After preincubation in Krebs-HEPES medium at 37°C for 10 min, the cells were stimulated by incubating them in high K^+ medium at 37°C for 6 min, and noradrenaline and adrenaline released during that time were determined (total release). Basal release of noradrenaline and adrenaline (data not shown) was determined by incubating cells in normal K^+ medium. When the effects of Ca^{2+} antagonists on catecholamine release were examined, Ca^{2+} antagonists were added to both preincubation and incubation medium. The data shown are high K^+-evoked catecholamine release, which was determined by subtracting basal release from total release: (●) NKY-722; (○) nicardipine; (◑) represents both NKY-722 and nicardipine. Data are means of three separate experiments, each done in triplicate; vertical bars show s.e.mean.

Figure 3 shows typical tracings illustrating the effects of NKY-722 on high K^+-evoked increases in $[Ca^{2+}]_i$ in suspended adrenal chromaffin cells. Basal $[Ca^{2+}]_i$ in the resting cells was unchanged upon addition of NKY-722 at a final concentration of 10^{-8} M or 10^{-6} M. In the absence of NKY-722, $[Ca^{2+}]_i$ increased from 0.15 μM to 0.40 μM (Figure 3a), when the cells were stimulated by high K^+. In the presence of 10^{-8} M NKY-722 (Figure 3b), the high K^+-evoked increase in $[Ca^{2+}]_i$ was reduced to about 50% of the value in its absence and completely inhibited at 10^{-6} M NKY-722 (Figure 3c). By comparing these data with those in Figure 2, the concentration-inhibition relationship for high K^+-evoked increases in $[Ca^{2+}]_i$ is consistent with that for high K^+-evoked $^{45}Ca^{2+}$ uptake. These data suggest that NKY-722 is acting mainly on Ca^{2+} channels to reduce the depolarization-induced increases in $[Ca^{2+}]_i$.

Figure 4 shows the effects of NKY-722 or nicardipine on high K^+-evoked release of noradrenaline and adrenaline from cultured adrenal chromaffin cells. Neither NKY-722 nor nicardipine had a significant effect on basal release of noradrenaline and adrenaline (data not shown). In contrast, high K^+-evoked release of noradrenaline was inhibited by NKY-722 or nicardipine in a concentration-dependent manner (Figure 4a). The IC_{50} values for high K^+-evoked noradrenaline release were 5.0 $\pm$ 0.3 nM ($n = 9$) for NKY-722 and 52 $\pm$ 2 nM ($n = 9$) for nicardipine. Essentially similar results were obtained for adrenaline release (Figure 4b): the IC_{50} values were 4.8 $\pm$ 0.3 nM ($n = 9$) for NKY-722 and 50 $\pm$ 3 nM ($n = 9$) for nicardipine. Again, the concentration-inhibition relationship for high K^+-evoked catecholamine release is in good agreement with that for high K^+-evoked $^{45}Ca^{2+}$ uptake and that for high K^+-evoked increases in $[Ca^{2+}]_i$. These results suggest that NKY-722, like nicardipine, reduces catecholamine release by inhibiting Ca^{2+} influx and a subsequent increase in $[Ca^{2+}]_i$.

To examine reversibility of the action of NKY-722, the cells were first preincubated in buffer containing various concentrations of NKY-722, and then stimulated by high K^+ in the drug-free buffer. Under these conditions, inhibition of high K^+-evoked release of noradrenaline and adrenaline was no longer observed (data not shown), suggesting that the effect of NKY-722 is reversible.

In summary, the present investigation established a novel, highly hydrophilic DHP compound, NKY-722, as a potent, reversible Ca^{2+} antagonist.

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The interaction between atrial natriuretic peptides and angiotensin II in controlling sodium and water excretion in the rat

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1 The present study was designed to determine how the natriuretic and diuretic actions of atrial natriuretic peptides were modulated by circulating angiotensin II.

2 In sodium pentobarbitone-anaesthetized rats, administration of bolus doses of atriopeptin III (1000 ng kg^{-1}) had no effect on blood pressure, renal blood flow, or glomerular filtration rate but caused reversible increases (all $P < 0.001$) in urine flow, of 53.9 ± 14.4 $\mu l\,kg^{-1}\,min^{-1}$, absolute sodium excretion, of 13.4 ± 2.9 $\mu mol\,kg^{-1}\,min^{-1}$ and fractional sodium excretion of 3.26 ± 0.74%. Similar effects were seen following a second dose of the atriopeptin III.

3 Following blockade of the renin-angiotensin system with captopril (900 $\mu g\,kg^{-1}\,h^{-1}$), control levels of blood pressure and haemodynamics were unchanged but there were significant (all $P < 0.001$) increases in urine flow, from 39.96 ± 5.05 to 88.70 ± 8.41 $\mu l\,kg^{-1}\,min^{-1}$, absolute sodium excretion, from 8.35 ± 1.08 to 21.62 ± 1.62 $\mu mol\,kg^{-1}\,min^{-1}$ and fractional sodium excretion, from 3.82 ± 0.23 to 5.34 ± 0.32%. Under these conditions, atriopeptin III-induced increases in urine flow (110.2 ± 8.7 versus 43.9 ± 6.2 $\mu l\,kg^{-1}\,min^{-1}$) absolute (24.0 ± 1.8 versus 9.3 ± 1.2 $\mu mol\,kg^{-1}\,min^{-1}$) and fractional ($5.16 \pm 0.24$ versus 2.08 ± 0.33%) sodium excretions were significantly ($P < 0.001$) greater.

4 In another group of rats given captopril, angiotensin II at 10 ng $kg^{-1}\,min^{-1}$ was also infused; this had no effect on blood pressure or renal haemodynamics, but partially restored basal levels of sodium and water excretion to those obtained before captopril. Atriopeptin III reversibly increased urine flow and absolute sodium excretion to the same degree as that obtained without captopril, but fractional sodium excretion was significantly larger than that obtained in the absence of captopril. In rats infused with angiotensin II at 15 ng $kg^{-1}\,min^{-1}$ together with the captopril the basal levels of fluid output were unchanged, while the magnitudes of the urine flow and sodium excretory responses to atriopeptin III were identical to those obtained before captopril and angiotensin II.

5 In animals subjected to two weeks of a low-sodium diet, atriopeptin III reversibly increased urine flow, absolute and fractional sodium excretions by between 53% and 74%; these responses were significantly ($P < 0.001$) smaller than those obtained in sodium replete rats. Administration of atriopeptin III, to low sodium diet rats given captopril, induced excretory responses which were significantly larger than those obtained in the absence of captopril.

6 The findings of this investigation demonstrate that in acute situations, angiotensin II exerts an important modulatory influence on the natriuretic potency of the atrial peptides by attenuating their action on the kidney. Long-term activation of the renin-angiotensin system depresses the renal excretory responses to atrial natriuretic peptides but suppression of angiotensin II production only partially restores the responsiveness of the kidney.

Keywords: atrial natriuretic peptides; angiotensin II; sodium excretion; renal function

Introduction

The atrial natriuretic peptides are released from the atria and act on the kidney to promote sodium and water excretion (Needleman *et al.*, 1989). The mechanisms underlying this action are unclear at present but a number of possibilities exist. An early finding was that atrial natriuretic peptides inhibited aldosterone release (Brenner *et al.*, 1990) but this would not account for the prompt and transient renal excretory responses. A further observation was that administration of high doses of atrial natriuretic peptides increased both renal blood flow and glomerular filtration rate, and one consequence would be increased filtered load which would tend to increase fluid output. Nevertheless, at low doses of atrial natriuretic peptides which have no renal haemodynamic action, there is still an impressive natriuretic and diuretic response (Brenner *et al.*, 1990) and this is now accepted as being due to a direct action of the peptides on tubular reabsorptive processes.

There is a high density of receptors for atrial natriuretic peptides at the glomerulus where they may be involved in the regulation of hydraulic conductivity via mesangial cell relaxation, whereas the evidence for receptors at the proximal tubules which may suppress tubular reabsorption is poor (Brenner *et al.*, 1990). By contrast, high affinity binding sites have been shown to exist in the inner medulla (Healy & Fanestil, 1986; Koseki *et al.*, 1986) and micropuncture studies have demonstrated that the atrial natriuretic peptides suppress fluid reabsorption along the cortical, medullary and papillary sections of the collecting tubules (Sonnenberg *et al.*, 1986). A consequence of such an action would be to diminish water retention and increase sodium excretion.

Recently, it has become apparent that one component of the tubular action of atrial natriuretic peptides could be due to its suppression of the action of angiotensin II at the proximal tubule where it normally stimulates sodium reabsorption (Harris & Skinner, 1990). Conversely, evidence is emerging that angiotensin II itself can inhibit the natriuretic action of atrial natriuretic peptides. Recently, Salazar *et al.* (1987) and Showalter *et al.* (1988) found that intrarenal infusion of angiotensin II in dogs blunted the natriuretic response to atrial natriuretic peptides when renal haemodynamics were

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Table 1 Responses in blood pressure and renal haemodynamic and excretory function to bolus doses of atriopeptin III (APIII, 1000 ng kg^{-1}) in sodium replete animals

		Control	*APIII 1*	*Control 2*	*APIII 2*
MAP (mmHg)	Group 1	116 ± 3	115 ± 4	115 ± 3	112 ± 3
	Group 2	113 ± 2	111 ± 2	112 ± 2	109 ± 3
	Group 3	118 ± 2	117 ± 3	116 ± 2	116 ± 2
	Group 4	114 ± 2	113 ± 2	113 ± 3	111 ± 3
RBF (ml kg^{-1} min^{-1})	Group 1	14.09 ± 0.84	14.32 ± 0.82	13.70 ± 0.16	13.88 ± 0.77
	Group 2	12.76 ± 1.38	12.38 ± 1.27	14.89 ± 1.80	14.89 ± 1.71
	Group 3	13.70 ± 0.83	14.39 ± 0.95	13.80 ± 0.72	13.89 ± 0.77
	Group 4	11.93 ± 1.27	11.81 ± 1.29	11.86 ± 1.39	11.42 ± 1.43
GFR (ml kg^{-1} min^{-1})	Group 1	2.81 ± 0.17	2.81 ± 0.22	2.70 ± 0.16	2.74 ± 0.19
	Group 2	2.53 ± 0.06	2.76 ± 0.08	2.52 ± 0.09	2.76 ± 0.14
	Group 3	2.40 ± 0.10	2.59 ± 0.13	2.17 ± 0.14	2.37 ± 0.09
	Group 4	2.67 ± 0.15	2.77 ± 0.11	2.48 ± 0.22	2.30 ± 0.27
UV (μl kg^{-1} min^{-1})	Group 1	52.55 ± 5.05	106.47 ± 11.69**	61.43 ± 9.58	121.06 ± 16.94**
	Group 2	39.96 ± 5.04	83.89 ± 10.88**	88.70 ± 8.41	198.86 ± 14.02**
	Group 3	67.90 ± 6.63	133.91 ± 14.85**	80.48 ± 5.45	148.97 ± 15.23**
	Group 4	55.94 ± 8.94	103.30 ± 15.97*	61.95 ± 7.89	98.79 ± 9.31*
$U_{Na}V$ (μmol kg^{-1} min^{-1})	Group 1	11.95 ± 1.05	25.35 ± 2.47**	15.00 ± 2.70	30.07 ± 4.32**
	Group 2	8.35 ± 1.08	17.67 ± 2.27**	21.62 ± 1.62	45.62 ± 2.28**
	Group 3	12.68 ± 1.20	24.12 ± 2.71**	16.06 ± 1.29	27.35 ± 1.47**
	Group 4	10.78 ± 1.94	20.36 ± 3.29*	13.41 ± 1.86	21.61 ± 2.54*
FE_{Na} (%)	Group 1	3.05 ± 0.40	6.31 ± 0.80**	3.43 ± 0.41	6.44 ± 0.72**
	Group 2	2.21 ± 0.29	4.29 ± 0.60**	5.65 ± 0.52	10.81 ± 0.57**
	Group 3	3.82 ± 0.23	6.79 ± 0.88**	5.34 ± 0.32	8.37 ± 0.60**
	Group 4	2.80 ± 0.47	5.02 ± 0.79*	3.77 ± 0.63	5.77 ± 0.88*

Group 1, normal saline infusion ($n = 6$); Group 2, captopril 900 μg kg^{-1} h^{-1} infused in the second part of the experiment ($n = 7$); Group 3, as Group 2, but angiotensin II 10 ng kg^{-1} min^{-1} infused with captopril ($n = 6$); Group 4, as Group 3, but angiotensin II at 15 ng kg^{-1} min^{-1} ($n = 6$). MAP, mean arterial pressure; RBF, renal blood flow; GFR, glomerular filtration rate; UV, urine flow, $U_{Na}V$, urinary sodium excretion, FE_{Na}, fractional sodium excretion. Control values 1 and 2 (first and second part of the experiment) represent the average values of the two clearances before and following atriopeptin III. APIII 1 and 2 represent the values achieved during atriopeptin III administration. n = number of animals.
Significantly different from control, *$P < 0.01$; **$P < 0.001$.

unchanged. Conversely, Bie *et al.* (1990) reported that blockade of angiotensin II production in the dog enhanced the action of ANP on renal fluid excretion, although in this study canrenoate was also used to block aldosterone activity.

The aim of this study was to determine whether the natriuretic response to the atrial natriuretic peptides is influenced by the circulating level of angiotensin II. This was done by comparing the increase in sodium excretion caused by atrial natriuretic peptides before and following the blockade of angiotensin II production with captopril. Further, the natriuretic effectiveness of atrial natriuretic peptides was determined when physiological levels of angiotensin II were restored by exogenous intravenous infusion, or elevated chronically subsequent to two weeks of a low sodium diet.

Methods

The experiments were performed on male Wistar rats (mean body weight 350 g) which had been fasted overnight. The animals were anaesthetized with pentobarbitone sodium, 60 mg kg^{-1} body weight i.p., and maintained with a constant infusion of pentobarbitone sodium (12 mg kg^{-1} h^{-1}) which was infused continuously until the end of the experiment. After the anaesthesia was induced, a tracheostomy was performed and catheters were placed in the right carotid artery for the measurement of arterial pressure and obtaining blood samples, and in the left jugular vein for infusions of saline and drugs. The intravenous infusion of isotonic saline (6 ml h^{-1}) was commenced immediately and continued for the duration of the experiment. The left kidney was exposed via a mid-line incision, its ureter cannulated for urine collection and the renal artery was carefully cleared and a flowmeter probe was then placed around the artery. Silver wire electrodes were applied onto the coeliac/aortico-renal ganglia and pulses of 15 V, 10 Hz and 0.2 ms were delivered for 10 s which caused a transient blanching of the kidney. If blanching did not occur, the animal was considered denervated and not included in the study. The arterial catheter was connected to a pressure transducer (Statham P231D) and the renal blood flow was measured with a square wave electromagnetic flowmeter (FM 501, Carolina Medical Instruments). Both arterial pressure and renal blood flow were continuously recorded on a polygraph (Model 7D Grass Instruments, U.S.A.). After surgery was completed a priming dose of 2 ml inulin in saline (1 g 100 ml^{-1}) was given intravenously and isotonic saline infusion was replaced with one containing inulin (1 g 100 ml^{-1}). A 2 h period of equilibration and stabilization was allowed before the clearance studies were performed.

Experimental protocols

Sodium replete animals The animals had free access to standard rat diet (sodium content 0.35%) and tap water. Ten clearance periods of 15 min each, were performed in two series of five clearances. Each series comprised two basal clearance periods followed by one experimental clearance, after which 15 min of recovery was allowed and then two recovery clearances were performed. Thirty min later the second series of clearances were undertaken. Arterial blood samples (300 μl each) were taken before the first and the third and after completion of the third and the fifth clearance periods and the same pattern was used in the second series of clearances. The blood samples were immediately centrifuged and plasma obtained, the red cells were resuspended in an equivalent volume of heparinized saline and reinfused into the animal. A 5 min equilibration period was allowed before the next collection period commenced. Urine was collected in pre-weighed microcentrifuge capped tubes.

Group 1 ($n = 6$) Control. Rat atriopeptin III (APIII, ANF5-28, Cambridge Research Biochemicals Limited, Cambridge, England) was given as a bolus dose of 1000 ng kg^{-1} in 0.3 ml normal saline intravenously 2 min before the experimental clearances.

Group 2 ($n = 7$) Animals were given APIII as in group 1. An infusion of captopril (900 μg kg^{-1} h^{-1}) was started after the

first series of clearances and continued throughout the rest of the experiment. The mean arterial pressure and renal blood-flow responses to angiotensin I 200 ng, given as a bolus dose in 0.2 ml saline, and angiotensin II 100 ng, as a bolus dose in 0.2 ml saline were obtained before the captopril infusion and at the end of the experiment at which time the responses to angiotensin I but not angiotensin II were abolished. The second series of clearances began 30 min after the start of captopril infusion.

Group 3 ($n = 6$) The experimental protocol was the same as that of group 2, but angiotensin II, 10 ng kg^{-1} min^{-1} was infused together with captopril.

Group 4 ($n = 6$) The same protocol was used as in group 3, but angiotensin II was infused at 15 ng kg^{-1} min^{-1} along with the captopril.

Low sodium animals The animals were placed on low sodium diet (Special Diet Services, Essex containing 0.05% sodium chloride) and distilled water two weeks before experiments. Only the first series of five clearances was performed in each animal according to the protocol described above. The animals were divided into two groups.

Low sodium (LS) group ($n = 6$) Animals received APIII in the same dose as sodium replete animals.

Low sodium animals receiving converting enzyme inhibitor (LS + CEI group) ($n = 5$) The infusion of captopril 900 μg kg^{-1} h^{-1} was started 30 min before the end of equilibrium period and continued throughout the experiment. The responses to bolus doses of angiotensin I and II were measured as described above.

Chemical assays

Urinary and plasma sodium concentration was measured by flame photometry (Corning 410C). Plasma and urine samples were deproteinised (Somogyi, 1930) and the inulin content assayed following the method of Bojesen (1952). Plasma inulin levels were taken as the average of the inulin values before and at the end of the paired or single clearance periods and glomerular filtration rate was calculated as inulin clearance.

Statistical analysis

All values represent means ± s.e.mean. The mean values of two basal and two recovery clearance periods were calculated for each series of five clearances and were considered as control values which were compared to the experimental value obtained during the influence of APIII. The statistical analysis was performed with two-way analysis of variance (ANOVA, FASTAT Software). The differences were taken to be significant when $P < 0.05$.

Results

Sodium replete study (Groups 1–4)

Table 1 presents the mean blood pressure and renal haemodynamic and functional variable responses to APIII administration for all experiments. Group 1 animals represent time controls and administration of APIII in the first half of the experiment had no effect on blood pressure, renal blod flow or glomerular filtration rate but significantly (all $P < 0.001$) and reversibly increased urine flow, by 53.9 ± 14.4 μl kg^{-1} min^{-1} absolute and fractional sodium excretions by 13.4 ± 2.9 μmol kg^{-1} min^{-1} and 3.26 ± 0.74%, respectively. In the second part of the study, control values of urine flow, absolute and fractional sodium excretion were slightly, but not significantly, raised and repeating this dose of APIII in the second part of the experiment gave a virtually identical pattern of excretory changes, the magnitudes of which could not be distinguished from those of the first part of the study. A comparison of these responses is shown in Figure 1.

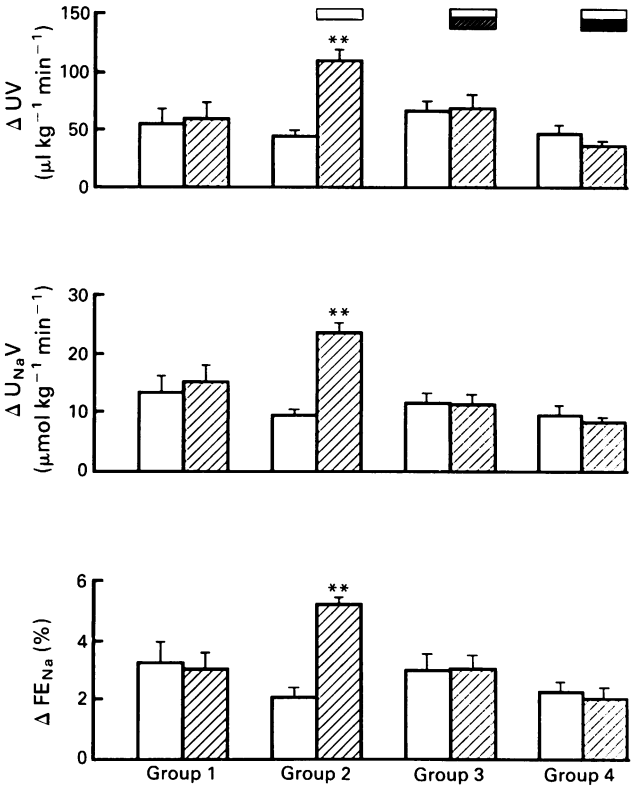

Figure 1 This shows the changes (Δ) in urine flow (UV), absolute ($U_{Na}V$) and fractional sodium excretion (FE_{Na}) from control levels in response to atriopeptin III (1000 ng kg^{-1}). The open histograms represent the responses to the first test with APIII, and the hatched histograms the responses observed following the second dose of APIII. Open horizontal bar: captopril, 900 μg kg^{-1} h^{-1}; hatched bar: angiotensin II, 10 ng kg^{-1} min^{-1}; solid bar: angiotensin II, 15 ng kg^{-1} min^{-1}.
**Significantly different from the first atriopeptin III challenge in that group.

In Group 2 animals (Table 1), the control values of all variables were similar to those of group 1 animals and administration of the first dose of APIII caused comparable reversible increases in urine flow, absolute and fractional sodium excretions (all $P < 0.001$). Infusion of captopril had no effect on blood pressure, renal blood flow or glomerular filtration rate, while control values of urine flow, absolute and fractional sodium excretion were all ($P < 0.001$) greater when compared to the first part of the experiment (Table 1). During the captopril infusion, the reversible increases in urine flow, absolute and fractional sodium excretions induced by APIII were all significantly ($P < 0.001$) larger than those obtained in the first part of the experiment (Figure 1) and those observed in the second part of the group 1 animals.

The animals of group 3 represent those in which exogenous angiotensin II was given together with the captopril (Table 1). The control values for blood pressure, renal haemodynamics excretory variables and excretory responses to APIII were very similar in both group 1 and 2 animals. In the second part of the experiment, angiotensin II was infused at 10 ng kg^{-1} min^{-1} together with the captopril and although this had no effect on the control values of urine flow or absolute sodium excretion, fractional sodium excretion was significantly ($P < 0.02$) raised. The administration of APIII under these conditions reversibly increased urine flow and absolute sodium excretion, the magnitudes of which could not be distinguished from those obtained either in the first half of the experiment or those obtained in group 1 in the second part of the experiment while the response in fractional sodium excretion (Figure 1) was significantly larger ($P < 0.02$).

Table 2 Responses in blood pressure and renal haemodynamic and excretory function to bolus doses of atriopeptin III (APIII) in low sodium animals

		Control	*APIII*
MAP	LS	107 ± 3	106 ± 3
(mmHg)	LS + CEI	105 ± 2	104 ± 2
RBF	LS	13.60 ± 0.92	14.02 ± 1.02
($ml\,kg^{-1}\,min^{-1}$)	LS + CEI	16.22 ± 1.27	16.15 ± 1.36
GFR	LS	2.35 ± 0.20	2.63 ± 0.23
($ml\,kg^{-1}\,min^{-1}$)	LS + CEI	3.77 ± 0.21	3.91 ± 0.26
UV	LS	28.97 ± 3.84	44.53 ± 5.57*
($\mu l\,kg^{-1}\,min^{-1}$)	LS + CEI	60.15 ± 2.39	102.46 ± 15.03*
$U_{Na}V$	LS	3.64 ± 0.74	6.37 ± 0.96**
($\mu mol\,kg^{-1}\,min^{-1}$)	LS + CEI	11.39 ± 0.85	21.09 ± 2.92**
FE_{Na}	LS	1.03 ± 0.16	1.62 ± 0.18**
(%)	LS + CEI	2.09 ± 0.12	3.68 ± 0.36**

LS, low sodium diet for two weeks ($n = 6$); LS + CEI, as LS but receiving captopril $900\,\mu g\,kg^{-1}\,h^{-1}$ i.v. from 30 min before experimental collections were started ($n = 5$). Abbreviations and significance values as in Table 1.

Group 4 animals were comparable to group 3, except that angiotensin was infused at a higher rate, $15\,ng\,kg^{-1}\,min^{-1}$, during the blockade of the renin-angiotensin system (Table 1). The control values of blood pressure and renal haemodynamics and excretory variables for the first part of the experiment were comparable to those obtained in the other groups over this period. There were no blood pressure or renal haemodynamic responses to APIII (Table 1) and the increases in urine flow, absolute and fractional sodium excretions (all $P < 0.001$) were very similar to all the other groups. The bolus dose of APIII during the co-administration of captopril with angiotensin II at $15\,ng\,kg^{-1}\,min^{-1}$ caused reversible increases in urine flow, absolute and fractional sodium excretions (all $P < 0.01$), which were similar in magnitude to those obtained in the first half of the experiment and those of the group 1 animals given the second dose of APIII (see Figure 1).

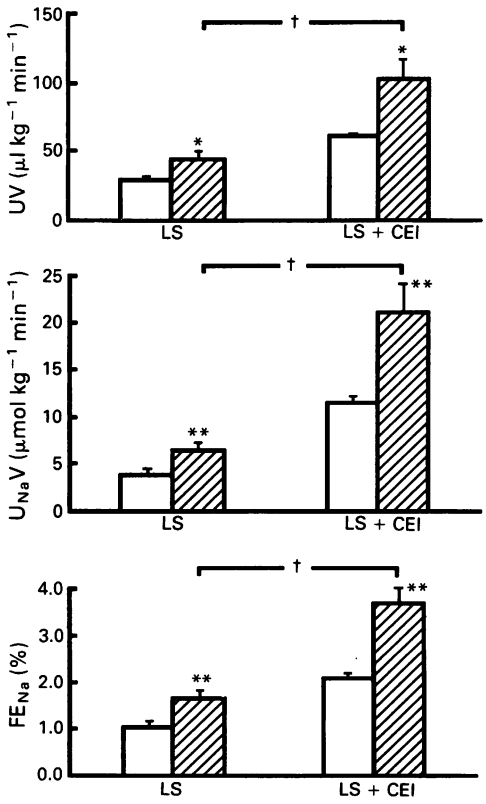

Figure 2 The responses in urine flow (UV), absolute ($U_{Na}V$) and fractional sodium excretion (FE_{Na}) to atriopeptin III (APIII) $1000\,ng\,kg^{-1}$ compared to control (mean of before and following APIII) in animals fed a low sodium diet for two weeks (LS) or also given captopril at $900\,\mu g\,kg^{-1}\,h^{-1}$ (LS + CEI). Open bars represent control values, hatched bars the values obtained after APIII bolus. Significantly different from control: *$P < 0.01$; **$P < 0.001$. Significantly different from values in LS group †$P < 0.001$.

Low sodium study

The control level of blood pressure in the low sodium diet animals (Table 2) was significantly lower than in the sodium replete animals ($P < 0.01$) and although both renal blood flow and glomerular filtration rate were similar to the replete groups, urine flow, absolute and fractional sodium excretions were all significantly ($P < 0.001$) lower. Administration of APIII to the animals fed a low sodium diet had no effect on blood pressure or renal haemodynamics but reversibly and significantly ($P < 0.001$) increased all excretory variables, the magnitudes of which were smaller when compared to the sodium replete animals (all $P < 0.001$).

Infusion of captopril into the animals fed low sodium diet (Table 2) had no effect on blood pressure, which remained lower than sodium replete animals, although renal blood flow was slightly raised. However, in these animals glomerular filtration rate was significantly ($P < 0.001$) greater as was urine flow, absolute and fractional sodium excretions (all $P < 0.001$). When the bolus dose of APIII was given, blood pressure and renal haemodynamics did not change but urine flow, absolute and fractional sodium excretions were significantly and reversibly elevated (all $P < 0.001$) and the responses in absolute and fractional sodium excretion were significantly (both $P < 0.05$) larger than those obtained in the rats subjected to the low sodium diet alone but were comparable to those obtained in the sodium replete animals (Figure 2).

Discussion

This study was undertaken to gain insight into the way in which angiotensin II could modulate the excretory responses of the kidney to atrial natriuretic peptides. We used a synthetic APIII at doses which had been shown in earlier studies to have no or minimal effects on renal haemodynamics (Johns & Rutkowski, 1989; 1990a,b). Under these conditions, the excretory responses obtained would be due primarily to a direct tubular action of the natriuretic peptide. In all animals we ensured that the innervation of the kidney was intact as there is evidence that renal nerve activity can blunt the excretory responses to the atrial natriuretic peptides, at least under some pathological situations (Koepke *et al.*, 1987).

The results from the first group of rats showed that the dose of $1000\,ng\,kg^{-1}$ of APIII caused an approximate doubling of both urine flow and sodium excretion comparable to the

effects reported earlier by ourselves (Johns & Rutkowski, 1989; 1990a,b) and Luft *et al.* (1986) using similar dose levels. These natriuretic and diuretic responses were most probably due to a direct tubular action of the peptide, possibly at some point along the tubule, collecting duct or medullary vasculature site (Harris & Skinner, 1990). It was clear from this time control group of rats that a second dose of ANP produced excretory responses which were similar in magnitude to those obtained to the first dose of peptide showing that the experimental preparation was stable and the results reproducible.

The contribution of angiotensin II, if any, to modulating the effectiveness of the ANP on the tubular responses of the kidney was approached initially by suppressing endogenous production of angiotensin II. Captopril was given in the second part of the study at $900\,\mu g\,kg^{-1}\,h^{-1}$ and at the end of the experiment was sufficient to abolish the vasopressor and renal vasoconstrictor response to a bolus dose of angiotensin I (200 ng) which was taken as good evidence that the renin-angiotensin system had been blocked and that circulating angiotensin II was at a low level. The captopril administration was associated with an increase in baseline levels of urine flow and sodium excretion which was most likely due to the removal of the antinatriuretic and antidiuretic action of angiotensin II (Johns, 1989). Administration of APIII in the presence of captopril led to an enhanced natriuretic and diuretic response compared to that obtained when the renin-angiotensin system was intact. The mechanism underlying this interaction of angiotensin II with APIII is unclear. One possibility could be that removal of the action of angiotensin II at the proximal tubule would result in a greater fluid load being presented to the more distal nephron segments, including the collecting duct, which would allow the APIII to act on a greater proportion of the fluid load, thereby generating a proportionately greater response.

These observations in the anaesthetized rat were similar to those reported by Bie *et al.* (1990) using the anaesthetized dog. They found that in animals given the aldosterone inhibitor, canrenoate and an intra-renal infusion of ANP there was an enhanced natriuresis following administration of a converting enzyme inhibitor. These findings were not supported by two recent papers describing experiments in conscious man (Gaillard *et al.*, 1988; Wamback *et al.*, 1989) in which the atrial natriuretic peptide-induced natriuresis was blunted following converting enzyme inhibition. The reasons for these differences remain to be resolved, and may be dependent on surgical stress activating the renin angiotensin system. However, in both human studies (Gaillard *et al.*, 1988; Wamback *et al.*, 1989) there was a concomitant reduction in blood pressure which would itself have acted to attenuate sodium excretion and possibly limited the response to the peptide.

In an attempt to implicate more clearly an action of angiotensin II, it was infused continuously during blockade of the renin-angiotensin system at a rate which was aimed to raise circulating levels to within the physiological range, but not such as to cause an increase in blood pressure or to reduce renal blood flow. Initially a rate of $10\,ng\,kg^{-1}\,min^{-1}$ angiotensin II was used but under these conditions the basal levels of sodium excretion were still somewhat elevated compared to the period before either captopril or angiotensin II. Nevertheless, the magnitude of the natriuretic and diuretic responses to APIII were suppressed compared to captopril alone and were similar to those obtained when captopril was not given. A second study was undertaken in which angiotensin II was infused at $15\,ng\,kg^{-1}\,min^{-1}$ in the presence of captopril and in this case basal levels of urine flow and sodium excretion were no different from those obtained in the absence of either compound; under these conditions the excretory responses to APIII were suppressed compared to those obtained when captopril was given alone, but were similar to those obtained in response to the first challenge with APIII. These studies complement and strengthen results of the initial study showing that it is indeed angiotensin II which attenuates the ability of the atrial natriuretic peptides to induce a natriuresis and diuresis. This viewpoint is supported by the findings of Salazar *et al.* (1987) and Showalter *et al.* (1988) using dogs in which angiotensin II, either in the presence or absence of an angiotensin convering enzyme inhibitor, appeared to limit the natriuretic effect of the atrial peptides. The exact sites within the kidney where these interactions might occur could not be assessed during the current approach. As proposed above, the action of angiotensin II at the proximal tubule would have reduced the amount of fluid presented to the collecting duct and thereby limited the magnitude of the response to the APIII.

The final study was an attempt to elevate chronically the levels of circulating angiotensin II by subjecting animals to two weeks of a low dietary sodium intake. The results clearly showed that under these conditions the magnitude of the natriuretic and diuretic responses were smaller compared to those obtained in sodium replete rats. Administration of the captopril to the low sodium rats resulted in higher levels of glomerular filtration rate and excretion of water and sodium than in its absence which was consistent with removal of elevated levels of angiotensin II (Johns, 1989). However, it was striking that when the renin-angiotensin system was blocked, although the natriuretic and diuretic responses to the atrial natriuretic peptides were enhanced, they only became comparable in magnitude to those obtained in the sodium replete animals. Indeed, it could be argued that the responses should have been of a similar size to those obtained in the replete rats receiving captopril. The reason for this not occurring is unclear, but may indicate that factors other than angiotensin II are involved under these conditions of chronic stimulation of the renin-angiotensin system. Indeed, Steele & Challoner-Hue (1990), using the isolated, buffer-perfused rat kidney, in which circulating angiotensin II was not present, found that the magnitude of the excretory responses to atrial natriuretic peptides were much less in rats subjected to four weeks of low sodium diet. These results and those of the present study implicate some factor, in addition to angiotensin II as being involved in modulating the renal excretory responses to atrial natriuretic peptide following low dietary sodium intake.

This study has attempted to define the role of angiotensin II in modulating the natriuretic and diuretic responses of the kidney to atrial natriuretic peptide. Blockade of the renin-angiotensin system enhanced, and exogenous infusion of angiotensin II suppressed, the natriuretic action of atriopeptin III. Further, the action of the atrial natriuretic peptide on the renal excretory responses was lower in the animals subjected to low sodium diet and was partially enhanced following converting enzyme inhibition. This study has shown that angiotensin II is an important factor which attenuates the action of atrial natriuretic peptides on the kidney and limits the magnitude of the natriuretic response. Under conditions of chronically elevated angiotensin II other factors appeared to come into play.

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Haemodynamic changes and acetylcholine-induced hypotensive responses after N^G-nitro-L-arginine methyl ester in rats and cats

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1 The haemodynamic effects of N^G-nitro-L-arginine methylester (L-NAME; 1, 3, 10 and 30 mg kg^{-1}) and its potential ability to attenuate the hypotensive responses to acetylcholine (0.03, 0.1, 1.0 and 3.0 μg kg^{-1}) have been investigated in anaesthetized rats and cats.

2 In the rat, L-NAME elicited a dose-dependent pressor effect increasing mean arterial blood pressure from the baseline value of 116 ± 4 mmHg to a maximum of 156 ± 6 mmHg with 30 mg kg^{-1}. This increase in blood pressure could be only partly reversed by L-arginine (300 mg kg^{-1}). However, the increase in blood pressure by lower doses (up to 10 mg kg^{-1}) of L-NAME was effectively reversed by L-arginine (1000 mg kg^{-1}).

3 In the cat, L-NAME did not significantly modify systemic haemodynamic variables (heart rate, mean arterial blood pressure, cardiac output, stroke volume or total peripheral resistance), when compared to the changes in saline-treated animals. Administration of L-arginine did not cause any significant effect in cats treated with L-NAME, but some decrease in heart rate and increases in cardiac output and stroke volume were observed in the saline-treated group.

4 With the lowest dose (1 mg kg^{-1}), L-NAME did not affect tissue blood flows in the cat, but higher doses (3 and 30 mg kg^{-1}) significantly reduced blood flows to the mesentery, stomach, spleen, intestines, lungs and the total liver. L-Arginine (300 mg kg^{-1}) injected into the control (saline-treated) animals resulted in a significant increase in blood flow to the heart, mesentery, lungs as well as the total liver, particularly its portal fraction. L-Arginine-induced increases in tissue blood flows (mesentery, kidneys, spleen, lungs, total liver and portal blood flow) in saline-treated animals were attenuated in animals treated with L-NAME.

5 The acetylcholine-induced peak hypotensive response was not reduced in rats or cats by L-NAME. The duration of acetylcholine response was, however, attenuated in both species by L-NAME. Treatment with L-arginine (10–100 mg kg^{-1}) did not change the acetylcholine-induced hypotension.

6 The above results reveal a marked difference between the haemodynamic effects of L-NAME in rats and cats and suggest that in cats, unlike rats, the role of the L-arginine-NO pathway in the regulation of blood pressure is rather limited, although such a pathway may exist in several tissues. Furthermore, the hypotensive response to acetylcholine in both species seems to be mediated largely by NO-independent pathways.

Keywords: Acetylcholine; L-arginine; endothelium; nitric oxide; N^G-nitro-L-arginine methyl ester.

Introduction

The vasodilator action of acetylcholine in a number of isolated blood vessels depends largely on the release of an endothelium-derived-relaxing factor (EDRF; Furchgott & Vanhoutte, 1989). EDRF has been characterized as being identical to nitric oxide (NO), which is cleaved from L-arginine by the action of the enzyme NO-synthase, present in endothelial cells (Palmer *et al.*, 1987; 1988). Once released, NO stimulates soluble guanylate cyclase to increase guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels (Boulanger *et al.*, 1990; Kelm & Schräder, 1990). The formation of NO can be inhibited *in vitro* by the arginine analogues, such as N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methylester (L-NAME) (Palmer *et al.*, 1988; Mülsch & Busse, 1990).

The local vasodilatation induced by acetylcholine is reported to be inhibited by L-NAME in the rat perfused mesentery (Moore *et al.*, 1990) and by L-NMMA in anaesthetized rats as well as in the human forearm arterial bed (Whittle *et al.*, 1989; Vallance *et al.*, 1989). In contrast, L-NAME has not been found to affect vasodepressor action in conscious rats (Gardiner *et al.*, 1990b). Since most experiments using arginine-analogues have been performed *in vitro*, we studied the systemic haemodynamic effects of L-NAME as well as its ability to modify the hypotensive responses to acetylcholine in rats and cats. In the latter animal species, tissue blood flow changes following the administration of L-NAME were also investigated.

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Methods

General

Rats Thirty-seven male Wistar-Kyoto rats (body weight 300–350 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.). A trachea canula was inserted to facilitate ventilation. Catheters were placed in both jugular veins and the right carotid artery for, respectively, drug administration and blood pressure measurement by a pressure transducer (model P23Ac, Statham Laboratories, Hato Rey, Puerto Rico). Blood pressure was continuously recorded on a polygraph (model 7, Grass Instrument Company, Quincey, MA). Heart rate was derived from the blood pressure recordings. Temperature was maintained at 37°C with an electric blanket.

Cats Twelve male or female cats (body weight between 2.8 and 5.0 kg) were anaesthetized with ketamine (12 mg kg^{-1}, i.p.). A trachea canula was inserted for artificial ventilation by a respiratory pump (Loosco, Amsterdam, The Netherlands).

Both femoral veins were catheterized for i.v. administration of drugs and blood sampling. Anaesthesia was maintained with sodium pentobarbitone (6 mg kg^{-1}, i.v. bolus, followed by 3 mg kg^{-1} h^{-1}, i.v. infusion). Both femoral arteries were cannulated for blood sampling and measurement of arterial blood pressure with a pressure transducer (model P23Ac, Statham Laboratories, Hato Rey, Puerto Rico). Blood pressure was continuously recorded on a polygraph (model 7, Grass Instrument Company, Quincy, MA). The right atrium was catheterized for microsphere administration. Finally, a flow probe was placed around the ascending aorta: cardiac output was calculated by adding ascending aorta blood flow and myocardial blood flow (see below). Body temperature was maintained at 37°C with an electric blanket and pH and blood gases were kept between normal limits (Po_2, 90–120 mmHg; Pco_2, 25–35 mmHg; pH, 7.35–7.45) by adjusting respiratory rate and tidal volume. A stabilization period of 30–60 min was allowed.

Regional tissue blood flows were measured with the radioactive microsphere technique, by use of the reference blood sample method (Heymann *et al.*, 1977; Saxena *et al.*, 1980). For each measurement, a suspension of about 200,000 microspheres (15 μm diameter, NEN Company, Dreieich, West Germany), labelled with one of the isotopes (^{114}Ce, ^{113}Sn, ^{103}Ru, ^{95}Nb, or ^{46}Sc), was mixed and injected into the left atrium. At the end of the experiment the animals were killed and various tissues were dissected out, weighed and put in vials. The radioactivity in these vials was counted for 5–10 min in a γ-scintillation counter (Packard, Minaxi Autogamma 5000) using suitable windows for discriminating the different isotopes. Regional blood flows and cardiac output were calculated with a set of computer programmes especially designed for the radioactive microsphere technique (Saxena *et al.*, 1980).

Experimental protocol

Rats After a stabilization period each rat received five increasing i.v. doses of acetylcholine (0.03, 0.1, 0.3, 1.0 and 3.0 μg kg^{-1}) at intervals of 3–5 min. Peak hypotensive responses to acetylcholine and its duration of action, i.e. the time needed for 50% recovery of the effect, were recorded. Due to the short-lasting action of acetylcholine, duration of action could only be assessed for the three highest doses. The rats were then divided into 4 groups which received either saline (4 times 1 ml kg^{-1}; $n = 9$), L-NAME (1, 3, 10 and 30 mg kg^{-1}; $n = 12$), L-arginine (10, 30 and 100 mg kg^{-1}; $n = 7$) or phenylephrine (40, 120, 220 and 330 μg kg^{-1}; $n = 9$). In most of the animals (for n, see results), the different doses of saline or drugs, administered every 15–20 min, were followed by the five doses of acetylcholine. The animals which had received L-NAME were administered L-arginine (300 mg kg^{-1}) after the last injection of acetylcholine. An additional group of animals ($n = 6$) was treated with L-NAME (1, 3 and 10 mg kg^{-1}) followed by L-arginine (100 mg kg^{-1}).

The dose range of phenylephrine was chosen to induce sustained increases in blood pressure similar to that induced with L-NAME. The magnitude and duration of hypotensive responses to acetylcholine after the different treatments were compared to the respective controls.

Cats A similar protocol was followed in cats as in rats for L-NAME (1, 3, 10 and 30 mg kg^{-1}) and saline. After the first series of acetylcholine injections (0.03, 0.1, 0.3, 1.0 and 3.0 μg kg^{-1}) at baseline, the first batch of microspheres was injected in each animal to determine baseline values of cardiac output and regional haemodynamic variables. The animals then received either four doses of L-NAME (1, 3, 10 and 30 mg kg^{-1}, $n = 7$) or saline (1 ml kg^{-1}, $n = 5$) at intervals of 20 min. Ten minutes after each dose (except after 10 mg kg^{-1} L-NAME and the third dose of saline), a batch of microspheres was injected. Finally, a bolus injection of 300 mg kg^{-1} L-arginine was administered, followed 10 min later by the last batch of microspheres for tissue blood flow measurements.

Data presentation and analysis

All data in the text are mentioned as mean ± s.e.mean. The values after the different treatments were compared to the baseline values by use of Duncan' new multiple range test once a parameteric two-way analysis of variance (randomized block design) had revealed that the samples represented different populations. The significance of the changes induced by L-NAME, L-arginine or phenylephrine as compared to the respective saline treatment was tested by an unpaired Student's t test. A P value of 0.05 or less was considered statistically significant.

Drugs

The drugs used in this study were: L-arginine hydrochloride, N^G-nitro-L-arginine methylester (both drugs: Sigma, St Louis, U.S.A.), ketamine hydrochloride (A.U.V., Cuyk, The Netherlands), acetylcholine chloride, phenylephrine hydrochloride (both from the Department of Pharmacy, Erasmus University, Rotterdam, The Netherlands), sodium pentobarbitone (Sanofi BV, Maassluis, The Netherlands). All drugs were dissolved in sterile saline. The doses mentioned in the text refer to the respective salts.

Results

Rats

Haemodynamic effects Both mean arterial blood pressure and heart rate remained relatively stable in the rats receiving either saline or L-arginine injections. Both L-NAME and phenylephrine elicited pressor responses; from the baseline values of 116 ± 4 and 93 ± 4 mmHg, respectively, blood pressure increased dose-dependently to a maximum of 156 ± 6 and 135 ± 5 mmHg, respectively, with the highest doses. Heart rate decreased significantly compared to baseline values with L-NAME, but the decreases were not different from those seen in saline-treated animals. Phenylephrine did not change heart rate (Figure 1). The increase in blood pressure induced by L-NAME (30 mg kg^{-1}) could be partly reversed by the administration of L-arginine (300 mg kg^{-1}) (Figure 1).

In an additional group of 6 animals which received L-NAME up to 10 mg kg^{-1}, blood pressure increased significantly from 93 ± 2 to a maximum of 142 ± 4 mmHg and heart rate decreased significantly from 336 ± 11 to 282 ± 13 beats min^{-1}. L-Arginine reversed the L-NAME-induced increase in blood pressure, which returned to a value of 78 ± 9 mmHg ($P < 0.05$, versus L-NAME 10 mg kg^{-1}). However, the bradycardia induced by L-NAME remained unchanged after L-arginine (288 ± 13 beats min^{-1}).

Acetylcholine responses Acetylcholine (0.03–3.0 μg kg^{-1}) elicited a dose-dependent fall in mean arterial blood pressure in rats; before any treatment the highest dose induced a fall in blood pressure of 52 ± 11 mmHg ($n = 25$). The calculated dose inducing a 40 mmHg decrease in mean arterial blood pressure (D_{40}) was 0.8 ± 0.1 μg kg^{-1} ($n = 25$). Treatment of rats with the four doses of saline did not influence the hypotensive responses to acetylcholine (Figure 2). Compared to the rats treated with saline, L-NAME (3 and 10 mg kg^{-1}) increased the hypotensive effects of 0.3 to 3.0 μg kg^{-1} acetylcholine. The vasodilator response to acetylcholine was also potentiated during infusions of phenylephrine (220 and 330 μg kg^{-1} h^{-1}). Pretreatment with L-arginine (10–100 mg kg^{-1}) did not change the acetylcholine-induced hypotension (Figure 2).

The duration of the depressor response to acetylcholine, assessed as the time needed for 50% recovery, remained unchanged after treatment with either saline, L-arginine or phenylephrine. There was, however, some significant reduction

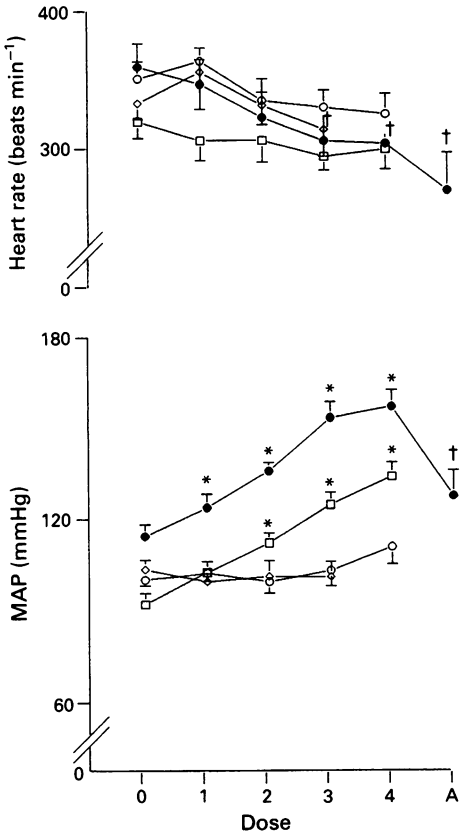

Figure 1 Mean arterial blood pressure (MAP) and heart rate in rats before (control; 0) and after treatment with four consecutive doses of saline (1 ml kg^{-1} each, $n = 9$; ○), N^G-nitro-L-arginine methylester (L-NAME, 1, 3, 10 and 30 mg kg^{-1}, $n = 12$; ●), phenylephrine (40, 120, 220 and 330 μg kg^{-1} h^{-1}, $n = 9$; □) or L-arginine (10, 30 and 100 mg kg^{-1}, $n = 7$; ◇). At A, L-arginine (300 mg kg^{-1}) was injected in the animals that had received L-NAME. Data represent mean with s.e.mean shown by vertical bars. Asterisks denote significant differences from the values in the saline experiments and crosses denote significant differences from baseline values, $P < 0.05$.

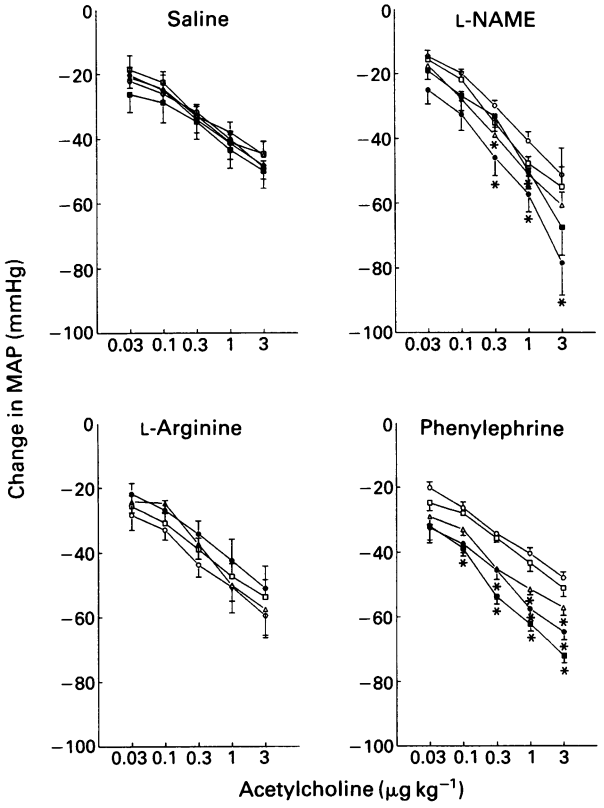

Figure 2 Decreases in mean arterial blood pressure (MAP) by acetylcholine (0.03–3.0 μg kg^{-1}) in rats before (control; ○) and after treatments with saline (1 ml kg^{-1} four times, $n = 8$), N^G-nitro-L-arginine methyl ester (L-NAME, 1, 3, 10 and 30 mg kg^{-1}, $n = 8$), phenylephrine (40, 120, 220 and 330 μg kg^{-1} h^{-1}, $n = 7$) or L-arginine (10, 30 and 100 mg kg^{-1}, $n = 7$). The increasing doses in each case are represented by the symbols (□), (△), (●) and (■), respectively. Data represent mean with s.e.mean shown by vertical bars. Asterisks denote significant differences from the values in the saline experiments, $P < 0.05$.

in the duration of action of acetylcholine (0.3 and 1.0 μg kg^{-1}) by L-NAME (Figure 3).

Cats

Haemodynamic effects The baseline values of the systemic and regional haemodynamic variables in the two groups of cats receiving, respectively, saline and L-NAME, both followed by L-arginine (300 mg kg^{-1}) are listed in Table 1. There were no significant differences between the two groups.

Figure 4 depicts the percentage changes in the systemic haemodynamic variables caused by saline (three injections) and L-NAME (1, 3 and 30 mg kg^{-1}). Treatment with saline did not cause any significant change other than a small decrease in heart rate. Similarly, the systemic haemodynamic effects of L-NAME in the cat were not marked. Although there was a small increase in total peripheral resistance (29 ± 5%) after 3 mg kg^{-1} L-NAME, these changes were only slightly more than those with the corresponding dose of saline (12 ± 5%). In the saline group, L-arginine decreased heart rate significantly (21 ± 3%), but cardiac output increased (12 ± 3%) due to an increase in stroke volume (42 ± 7%) (Figure 4). Administration of L-arginine did not cause any significant effect in cats treated with L-NAME, but the changes observed in the saline group appeared to be blunted (Figure 4).

The effects of L-NAME (1, 3 and 30 mg kg^{-1}) on tissue blood flows in the cat are shown in Figure 5. With the lowest dose no changes were observed, but the higher doses of L-NAME significantly reduced blood flows to several tissues including the mesentery, stomach, spleen, intestines, lungs and the total liver (Figure 5). L-Arginine (300 mg kg^{-1}) injected into the control (saline-treated) animals resulted in a significant increase in blood flow to the heart, mesentery, lungs as well as the total liver, particularly its portal fraction (Figure

Table 1 Baseline values of systemic haemodynamic variables and organ blood flow in anaesthetized cats treated with saline ($n = 5$) or N^G-nitro-L-arginine methyl ester (L-NAME) ($n = 7$)

	Saline	*L-NAME*
Systemic haemodynamic variables		
Mean arterial blood pressure (mmHg)	101.2 ± 6.1	95.1 ± 5.6
Heart rate (beats min^{-1})	153.0 ± 4.9	143.3 ± 14.6
Stroke volume (ml)	3.3 ± 0.3	3.9 ± 0.8
Cardiac output (ml min^{-1})	503.8 ± 42.1	512.7 ± 76.9
Total peripheral resistance (mmHg l^{-1} min^{-1})	203.5 ± 11.4	214.4 ± 29.0
Organ blood flow (ml min^{-1})		
Heart	13.5 ± 5.0	10.1 ± 1.5
Brain	8.5 ± 1.8	7.1 ± 1.1
Lungs	39.9 ± 5.7	39.8 ± 5.2
Stomach	8.0 ± 2.1	7.2 ± 1.7
Intestine	27.8 ± 6.0	25.6 ± 3.3
Spleen	14.0 ± 4.6	7.1 ± 1.6
Mesentery	7.6 ± 2.6	5.2 ± 1.1
Portal	68.7 ± 16.4	58.4 ± 9.5
Total liver	86.2 ± 23.7	71.0 ± 8.9
Kidneys	39.2 ± 5.9	38.5 ± 4.4
Muscle	3.8 ± 0.9	3.4 ± 0.4
Skin	1.4 ± 0.2	1.5 ± 0.4

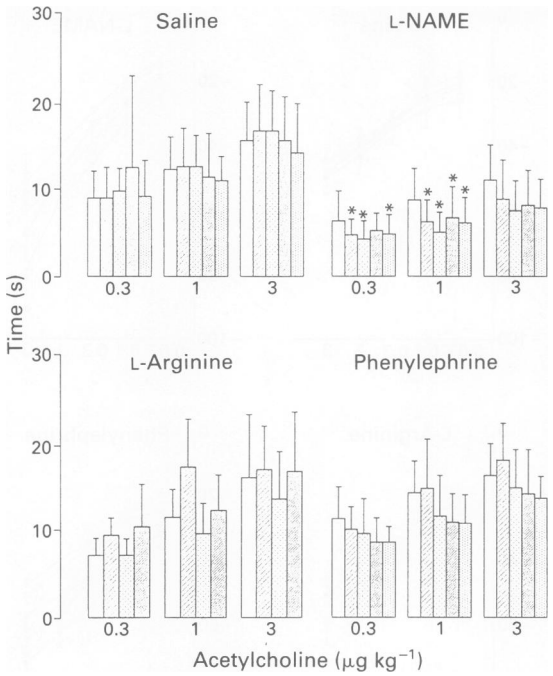

Figure 3 Time needed for 50% recovery in the hypotensive action of acetylcholine (0.3, 1 and 3 $\mu g\,kg^{-1}$) in rats treated with saline (1 $ml\,kg^{-1}$ four times, $n = 8$), N^G-nitro-L-arginine methyl ester (L-NAME, 1, 3, 10 and 30 $mg\,kg^{-1}$, $n = 8$), L-arginine (10, 30 and 100 $mg\,kg^{-1}$, $n = 7$) or phenylephrine (40, 120, 220 and 330 $\mu g\,kg^{-1}\,h^{-1}$, $n = 7$). In each set of panels, the 5 columns (4 in case of L-arginine) represent, sequentially, the responses before and after the increasing doses of saline or drugs. Data represent mean with s.e.mean shown by vertical bars. Asterisks denote significant differences from the values in the saline experiments, $P < 0.05$.

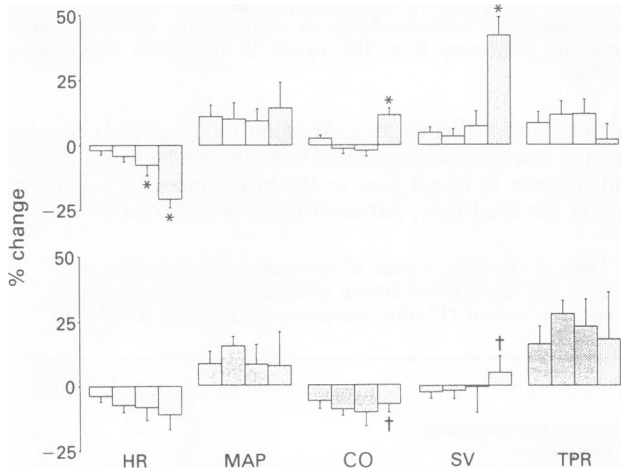

Figure 4 Percentage changes from baseline in systemic haemodynamic values in cats treated with saline (1 $ml\,kg^{-1}$ three times, $n = 5$) or N^G-nitro-L-arginine methyl ester (L-NAME, 1, 3 and 30 $mg\,kg^{-1}$, $n = 7$). Data after the 3rd dose of saline or L-NAME (10 $mg\,kg^{-1}$), where no microspheres were given (see Methods), were not collated. In each set of panels, the first three columns represent, sequentially, the increasing doses of saline or L-NAME, while the fourth column represents L-arginine (300 $mg\,kg^{-1}$). Abbreviations: heart rate (HR), mean arterial blood pressure (MAP), cardiac output (CO), stroke volume (SV) and total peripheral resistance (TPR). Data represent mean with s.e.mean shown by vertical bars. Asterisks denote significant changes compared to baseline values, crosses denote significant changes compared to saline experiments, $P < 0.05$.

5). The changes induced by L-arginine in animals treated with L-NAME were significantly different from the changes in saline-treated animals in the mesentery, kidneys, spleen, lungs as well as the total liver and portal blood flow (Figure 5).

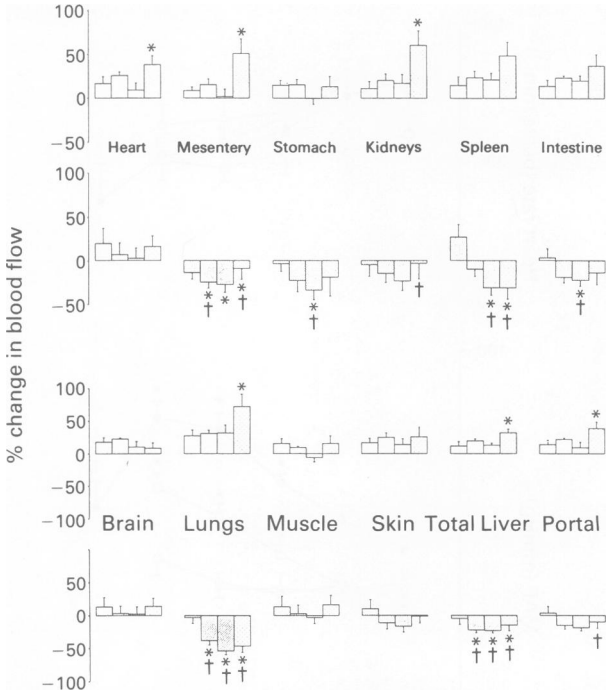

Figure 5 Percentage changes from baseline in tissue blood flows in cats treated with saline (1 $ml\,kg^{-1}$ three times, $n = 5$) or N^G nitro-L-arginine methyl ester (L-NAME, 1, 3 and 30 $mg\,kg^{-1}$, $n = 7$). As mentioned in Methods, tissue blood flows were not measured after the 3rd dose of saline or L-NAME (10 $mg\,kg^{-1}$). In each set of panels, the first three columns, sequentially, represent the increasing doses of saline or L-NAME, while the fourth column represents L-arginine (300 $mg\,kg^{-1}$). Data represent mean with s.e.mean shown by vertical bars. Asterisks denote significant changes compared to baseline values, crosses denote significant changes compared to saline experiments, $P < 0.05$.

Acetylcholine responses Acetylcholine induced a dose-dependent hypotensive effect in anaesthetized cats in doses ranging from 0.03 to 3.0 $\mu g\,kg^{-1}$. The fall in mean arterial blood pressure after the highest dose was 62 ± 5 mmHg ($n = 9$). The calculated dose of acetylcholine inducing a 40 mmHg decrease in mean blood pressure (D_{40}) was $0.11 \pm 0.04\,\mu g\,kg^{-1}$ ($n = 9$). L-NAME (1, 3, 10 and 30 $mg\,kg^{-1}$) did not significantly affect the fall in blood pressure induced by acetylcholine (Figure 6). However, L-NAME (from 3 $mg\,kg^{-1}$ and higher) was able to reduce the duration of action of acetylcholine; the time needed for 50% recovery in the acetylcholine responses was clearly reduced (Figure 7).

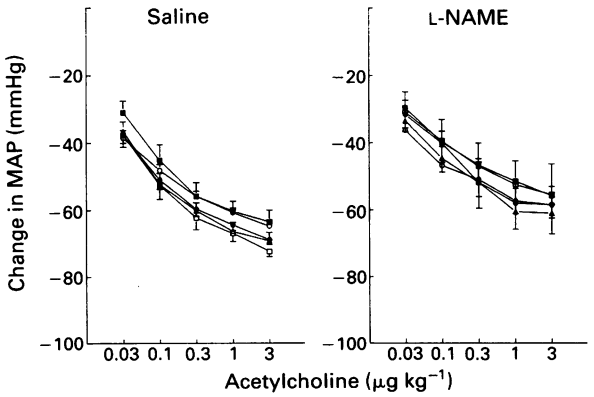

Figure 6 Decreases in mean arterial blood pressure (MAP) by acetylcholine (0.03–3.0 $\mu g\,kg^{-1}$) in cats before (control; ○) and after treatments with saline (1 $ml\,kg^{-1}$ four times, $n = 5$) or N^G-nitro-L-arginine methyl ester (L-NAME, 1, 3, 10 and 30 $mg\,kg^{-1}$, $n = 6$). The increasing doses in each case are represented by the symbols (□), (△), (●) and (■) respectively. Data represent mean with s.e.mean shown by vertical bars. No significant differences between saline and L-NAME experiments were observed.

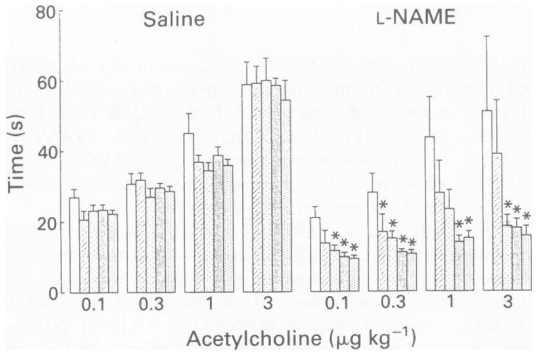

Figure 7 Time needed for 50% recovery in the hypotensive action of acetylcholine (0.3–3.0 $\mu g\,kg^{-1}$) in cats treated with saline (1 $ml\,kg^{-1}$ four times, $n = 5$) or N^G-nitro-L-arginine methyl ester (L-NAME, 1, 3, 10 and 30 $mg\,kg^{-1}$, $n = 6$). In each set of panels, the 5 columns represent, sequentially, the responses before and after increasing doses of saline or L-NAME. Data represent mean with s.e.mean shown by vertical bars. Asterisks denote significant differences from the values in the saline experiments, $P < 0.05$.

Discussion

Haemodynamic responses

Our findings that L-NAME, when injected in pentobarbitone-anaesthetized rats, induced a significant dose-dependent increase in blood pressure and reversal by L-arginine is consistent with results found in conscious as well as anaesthetized rats (Gardiner *et al.*, 1990a,b,c; Rees *et al.*, 1990). However, the concomitant fall in heart rate reported in anaesthetized rats by Rees *et al.* (1990) was comparatively less marked with L-NAME in our experiments as heart rate also decreased in animals treated with saline only. Administration of L-arginine, particularly when the dose was higher (100 fold) than that of L-NAME (see also Rees *et al.*, 1990), reversed the increases in blood pressure induced by L-NAME.

In contrast to rats, surprisingly, no significant changes in mean arterial blood pressure were found with four consecutive doses of L-NAME (1, 3, 10 and 30 $mg\,kg^{-1}$) in cats anaesthetized with pentobarbitone and ketamine (initially). This inability of L-NAME to elicit hypertension has also been observed in cats anaesthetized with α-chloralose and pentobarbitone (R. Saxena, E. Scheffers & R. Shukla, unpublished). Though L-NAME had little overall effect on haemodynamics, the drug did elicit a dose-dependent and significant decrease in blood flow to and, therefore, an increase in vascular resistance in several tissues (lungs, liver, stomach, small intestine and mesentery), suggesting an inhibition of the release of NO by L-NAME in these organs. Administration of L-arginine in cats, treated with L-NAME, did not reverse the decrease in blood flow induced by L-NAME. In saline-treated animals, however, L-arginine revealed a significant increase in blood flow in the lungs, mesentery, liver and several other organs, including the heart and the kidneys. The above results suggest that in cats there seems to be a difference in the basal release of NO; some organs, like the lungs, liver and mesentery, show more basal release than other tissues like the heart and kidneys. The findings that neither L-NAME nor L-arginine changed the mean arterial blood pressure and that L-NAME induced a decrease in blood flow in only a few organs suggest little contribution of the L-arginine-NO pathway to blood pressure regulation in the anaesthetized cat.

Although L-arginine increased blood flow in several organs, the total peripheral resistance did not change. This can be due to a concomitant increase in cardiac output, following an enhanced stroke volume due to an improved cardiac filling. Gardiner *et al.* (1990c) suggest that the decrease in cardiac output induced by L-NAME in rats could be due to a direct negative inotropic action or due to a coronary vasoconstriction. Indeed, L-NAME can induce coronary vasoconstriction in conscious rabbits (Humphries *et al.*, 1991). Our experiments in cats, however, show that despite a significant increase in myocardial blood flow by L-arginine, L-NAME neither decreased coronary artery blood flow nor decreased cardiac output. This again suggests that, unlike the rat, there is little spontaneous release of NO from the cat heart.

Acetylcholine responses

There seems to be little doubt that the vascular smooth muscle relaxation *in vitro* or local vasodilatation in several vascular beds following acetylcholine administration in different species is largely dependent on the release of NO and is, consequently, inhibited by drugs like L-NAME (Aisaka *et al.*, 1989; Furchgott & Vanhoutte, 1989; Vallance *et al.*, 1989; Whittle *et al.*, 1989; Kontos *et al.*, 1990; Moore *et al.*, 1990). However, it has also been reported that the hypotensive response to acetylcholine in conscious rats is not amenable to blockade by L-NAME (Gardiner *et al.*, 1990b). Our results, both in anaesthetized rats and cats, are in agreement with the latter findings. Indeed, the hypotensive effect of acetylcholine was even potentiated in the rat after treatment with 10 $mg\,kg^{-1}$ L-NAME. This apparent potentiation was due to the increase in arterial pressure by L-NAME, since it was also observed after phenylephrine infusions in the rat, but not during L-NAME treatment in the cat where arterial pressure was not affected by the drug. Therefore, it seems likely that the magnitude of the hypotension elicited by acetylcholine *in vivo* is not dependent on the release of NO. It may, however, be noted that the highest dose of L-NAME (30 $mg\,kg^{-1}$) did not further increase the depressor responses to acetylcholine in rats; on the contrary the magnitude of the responses were not significantly different from those in saline-treated animals (see Figure 2), suggesting that there may be some inhibition of the acetylcholine depressor effects in rats at the highest dose of L-NAME.

Despite little change in the magnitude of the hypotensive response to acetylcholine, there was a significant reduction in its duration by L-NAME in both cats and rats; phenylephrine infusion in the rat had no effect. In contrast to experiments in guinea-pigs with L-NMMA (Aisaka *et al.*, 1989), L-arginine did not prolong the duration of the acetylcholine response in our experiments. Nevertheless, our findings do support the suggestions of Aisaka *et al.* (1989) that the first phase of the depressor response to acetylcholine is NO-independent. It is tempting to speculate that the second phase is, in part, due to a hyperpolarization of the vascular smooth muscle resulting from the release of an endothelium-dependent-hyperpolarizing-factor (EDHF). Indeed, in dog mesenteric arteries the hyperpolarization induced by acetylcholine, in contrast to the relaxation, seems to be NO-independent, since oxyhaemoglobin and methylene blue prevented only the latter (Komori *et al.*, 1988). However, in guinea-pig isolated uterine arteries L-NMMA blocked both hyperpolarization and relaxation responses to acetylcholine, suggesting that EDHF may be identical to NO in this species (Tare *et al.*, 1990).

Lastly, it may be mentioned that acetylcholine, when applied topically, induces cerebral dilatation in cats by releasing an EDRF-like substance (Kontos *et al.*, 1990). Our experiments, however showed no significant increase in brain blood flow by L-arginine or a decrease by L-NAME. These findings may indicate that, unlike large conducting vessels, there is only a limited contribution of EDRF/NO to cerebral blood flow regulation.

In conclusion, the present *in vivo* experiments reveal a marked difference between the effects of L-NAME in rats and cats; in contrast to rats, the contribution of the L-arginine-NO pathway to blood pressure regulation in the cat seems to be very limited, although such a pathway seems to exist in several tissues. In addition, L-NAME does not affect the magnitude of hypotensive response to acetylcholine in either species, but reduces its duration.

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Nitroglycerin relaxes coronary artery of the pig with no change in glutathione content or glutathione S-transferase activity

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1 The role of glutathione content and glutathione S-transferase activity in vascular relaxant responses to nitroglycerin was evaluated in potassium (30 mM)-contracted coronary artery strips of the pig by measuring changes in tension, glutathione content and glutathione S-transferase activity.

2 Prior exposure of coronary artery strips to nitroglycerin (10^{-5} M or 10^{-4} M for 20 min) resulted in tachyphylaxis to subsequent relaxation to nitroglycerin (10^{-8}–10^{-5} M).

3 The glutathione content and glutathione S-transferase activity of the arterial strips rendered tachyphylactic by prior exposure to nitroglycerin (10^{-5} M for 20 min or 10^{-3} M for 120 min) were not significantly different from those of control strips.

4 Treatment with diethyl maleate (10^{-4} M or 10^{-3} M for 60 min) markedly depleted arterial glutathione content in a concentration-dependent manner with no change in glutathione S-transferase activity.

5 The relaxant response of coronary artery strips to nitroglycerin (10^{-8}–10^{-5} M) was completely unaffected following treatment with diethyl maleate (10^{-4} M or 10^{-3} M for 60 min).

6 The results suggest that vascular glutathione content does not play an important role in vascular relaxation or tolerance development to nitroglycerin, at least in pig isolated coronary artery.

Keywords: Nitroglycerin; glutathione; glutathione S-transferase; diethyl maleate; tolerance; vasorelaxation; porcine coronary artery.

Introduction

It has been proposed that organic nitrates produce vascular smooth muscle relaxation resulting from generation of S-nitrosothiols or nitric oxide and that these processes require the presence of glutathione and glutathione S-transferase. The nitric oxide or S-nitrosothiols thus formed then activate soluble guanylate cyclase producing guanosine 3′:5′-cyclic monophosphate and vascular relaxation (Needleman *et al.*, 1973; Ignarro *et al.*, 1981; Ignarro & Kadowitz, 1985; Kukovetz & Holzmann, 1985). Moreover, it has been proposed that the development of tolerance following repetitive application of nitroglycerin is due to depletion of vascular glutathione, thus preventing enzymic conversion of the nitrovasodilator to the active species (Needleman & Johnson, 1973; Neeldman *et al.*, 1973). This speculation is supported by the finding that the sulphydryl compound, *N*-acetylcysteine, reverses tolerance to nitrovasodilators in vascular smooth muscle by restoring glutathione content (Torresi *et al.*, 1985; Winniford *et al.*, 1986; Packer *et al.*, 1987; Svendsen *et al.*, 1989). Thus, glutathione consumption is considered to be needed for both vascular smooth muscle relaxation and tolerance development to nitrates. Despite this, however, only one study has been described in which measurement of vascular glutathione content has been performed in association with vascular relaxation and tolerance development (Gruetter & Lemke, 1985). The present work, therefore, was designed to clarify the relationship between vascular relaxation, tolerance development, and vascular glutathione content. In this study, relaxant responses to nitroglycerin and changes in glutathione content induced by nitroglycerin treatment were examined in isolated coronary arteries of the pig. In addition, the effects of diethyl maleate, which is known to deplete glutathione (Boyland & Chasseaud, 1967; 1968), were observed on relaxant responses and changes in glutathione content. Furthermore, glutathione S-transferase activity was also measured to clarify the role of this enzyme in vascular relaxation.

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Methods

Preparation of coronary artery rings

After slaughter, hearts were removed from pigs of either sex weighing 70–100 kg and placed in ice-cold Krebs-Henseleit solution of the following composition (mM): NaCl 120, KCl 4.8, $CaCl_2$ 1.25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11. The proximal part of the left circumflex coronary artery was cut into ring preparations of 2–3 mm diameter and 1–1.5 mm wide. These arterial preparations were not denuded of endothelium, and thus the present study was carried out on the whole coronary artery with endothelium. The ring preparations were suspended in organ baths filled with Krebs-Henseleit solution maintained at 37°C and gassed with 95% O_2 and 5% CO_2. One end of the arterial rings was fixed at the base of the bath, and the other was connected to an isometric force-displacement transducer (Nihon Kohden, TB-611T, Tokyo, Japan) for measurement of changes in tension. The resting tension of the rings was adjusted to 1.0 g. Changes in tension were recorded on an ink-writing recorder (Nihon Kohden, RJG-4004).

Experimental protocol

After 90 min of equilibration time, KCl 30 mM was added to test the viability of the preparations and then washed out. Fifteen min later, nitroglycerin or Krebs-Henseleit solution (vehicle) was added and the rings were incubated for 20 min, before the bathing solution was exchanged for fresh Krebs-Henseleit solution. Twenty min after exchanging the bathing solution, KCl 30 mM was added to the baths again. After stabilization of the potassium-induced contracture, nitroglycerin was added cumulatively. Relaxant responses to nitroglycerin were represented as the percentage relaxation when the potassium (30 mM)-induced contracture was defined as 100% (100% = 1.8 ± 0.1 g in vehicle-incubated preparations as controls or 1.5 ± 0.2 g in nitroglycerin-incubated preparations, respectively).

In another series of experiments, the preparations were preincubated with diethyl maleate for 60 min and washed with fresh Krebs-Henseleit solution. KCl 30 mM was added to test the viability of the rings, and then washed out. Fifteen min after washing the preparations, KCl 30 mM was applied again, and after stabilization of the induced contracture, nitroglycerin was added cumulatively. In this series of experiments, too, relaxant responses to nitroglycerin were represented as the percentage relaxation when the potassium (30 mM)-induced contracture was defined as 100% (100% = 2.1 ± 0.1 g in vehicle-incubated preparations as controls or 1.9 ± 0.2 g in diethyl maleate-incubated preparations, respectively).

Measurement of glutathione content and glutathione S-transferase activity

Small segments (5–10 mm) of coronary artery were collected from 5 pig hearts and equilibrated with Krebs-Henseleit solution for 90 min at 37°C. The arteries were then incubated with nitroglycerin 10^{-5} M for 20 min or with nitroglycerin 10^{-3} M for 120 min, or with diethyl maleate 10^{-4} M or 10^{-3} M for 60 min. The artery segments were washed 3 times with Krebs-Henseleit solution and homogenized in 5 volumes of 0.9% NaCl solution with a polytron homogenizer (Kinematch, Switzerland). After centrifugation at 9000 *g* for 30 min at 4°C, the supernatant was retained. The protein content was measured by the method of Lowry *et al.* (1951). Glutathione S-transferase activity was measured by the method of Habig *et al.* (1974) using 1-chloro-2,4-dinitrobenzene 1 mM and reduced glutathione 1 mM as substrates, and the glutathione content was measured by the method of Reed *et al.* (1980) and Gruetter & Lemke (1985) with a Toyo Soda high-performance liquid chromatograph (CCPM; Tokyo, Japan), a UV 8000 detector using iodoacetic acid (4 μM), an excess of $NaHCO_3$ (dry powder) for neutralization and an alcoholic solution of 1-fluoro-2,4-dinitrobenzene (1.5 ml/98.5 ml absolute ethanol) as the reaction solvent.

Drugs

Drugs used in this study were nitroglycerin (Millisrol; Nihonkayaku, Tokyo, Japan), diethyl maleate (Sigma, St. Louis, U.S.A.), reduced glutathione (Sigma), 1-chloro-2,4-dinitrobenzene (Wako, Tokyo, Japan), iodoacetic acid (Sigma), $NaHCO_3$ (Wako) and 1-fluoro-2,4-dinitrobenzene (Wako). All other chemicals were of analytical reagent grade.

Statistics

Data were analyzed by Student's *t* test, and a significant difference was assumed when a *P* value was less than 5%.

Results

Potassium chloride, 30 mM, contracted pig coronary artery strips to a level of 1.8 ± 0.1 g ($n = 26$). In 14 preparations which were previously incubated with Krebs-Henseleit solution as the control, nitroglycerin 10^{-8}–10^{-5} M produced concentration-dependent relaxations as shown in Figure 1. In 7 preparations which were previously incubated with nitroglycerin 10^{-5} M for 20 min, the concentration-relaxation curve for nitroglycerin 10^{-8}–10^{-5} M was significantly shifted to the right. Prior incubation with nitroglycerin 10^{-4} M for 20 min ($n = 7$) shifted the concentration-relaxation curve for nitroglycerin 10^{-8}–10^{-5} M even further to the right (Figure 1). Thus, prior incubation with nitroglycerin resulted in the development of tachyphylaxis.

No change in arterial glutathione content or glutathione S-transferase activity was observed following incubation at 37°C with nitroglycerin 10^{-5} M for 20 min, or 10^{-3} M for 120 min (Table 1).

In order to evaluate further the relationship between vascular relaxation and glutathione content, the effects of the glutathione depleting agent, diethyl maleate, were examined.

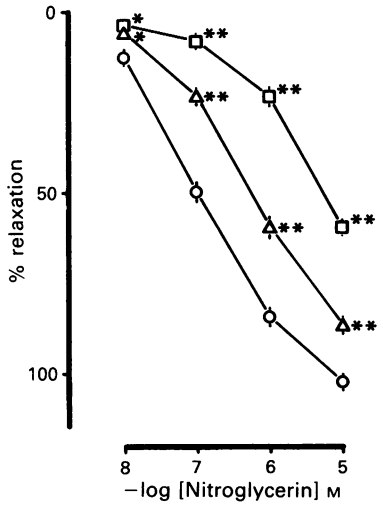

Figure 1 The relaxant effects of nitroglycerin on pig isolated coronary artery rings following contraction with potassium chloride (30 mM). Controls (○); preparations previously incubated with nitroglycerin 10^{-5} M for 20 min (△); preparations previously incubated with nitroglycerin 10^{-4} M for 20 min (□). Each value represents the mean percentage relaxation ($n = 7$–14) of potassium-induced tone; vertical lines show s.e.mean.

* $P < 0.05$ and ** $P < 0.01$ indicate a difference from untreated tissues.

Table 1 Effect of nitroglycerin (NG) on glutathione content (GSH) and glutathione S-transferase activity (GST) in pig coronary artery

Incubation	n	*GSH* (nmol mg^{-1} protein)	*GST* (nmol mg^{-1} protein min^{-1})	
Control	5	18.2 ± 5.5	45.9 ± 6.0	37°C, 20 min
NG 10^{-5} M	5	17.2 ± 4.8	44.4 ± 4.7	incubation
Control	5	14.7 ± 2.9	36.3 ± 7.1	37°C, 120 min
NG 10^{-3} M	5	14.4 ± 4.3	35.3 ± 7.1	incubation

Each value is the mean ± s.d.

Table 2 Effect of diethyl maleate (DEM) on glutathione content (GSH) and glutathione S-transferase activity (GST) in pig coronary artery

Incubation	n	*GSH* (nmol mg^{-1} protein)	*GST* (nmol mg^{-1} protein min^{-1})	
Control	5	14.3 ± 1.0	47.1 ± 1.7	37°C, 60 min
DEM 10^{-4} M	5	7.7 ± 2.3***	48.1 ± 2.7	incubation
DEM 10^{-3} M	5	0.9 ± 0.6***	49.3 ± 4.7	

Each value is the mean ± s.d.

*** $P < 0.001$ vs. the corresponding control values.

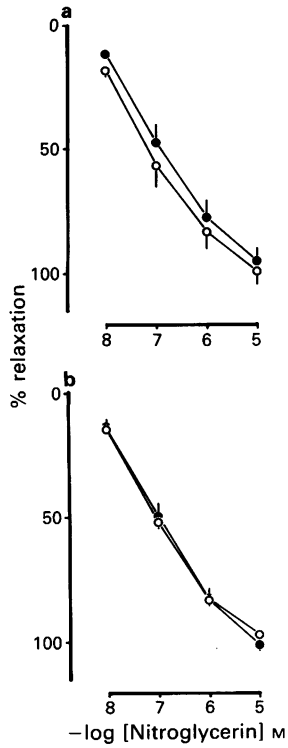

Figure 2 Effects of prior exposure to diethyl maleate on the relaxant response of potassium (30 mM)-contracted pig coronary artery rings to nitroglycerin. Controls (○); preparations previously incubated with diethyl maleate (●) at 10^{-4} M (a) and 10^{-3} M (b). Each value represents the mean percentage relaxation ($n = 6$) of potassium-induced tone; vertical lines show s.e.mean.

Diethyl maleate 10^{-4} M and 10^{-3} M significantly decreased the glutathione content to about 45% and 5%, respectively, of the control level after 60 min incubation, with no change in glutathione S-transferase activity (Table 2). The decrease in glutathione content with no change in glutathione S-transferase activity persisted for at least 120 min after the 60 min incubation with diethyl maleate 10^{-4} M or 10^{-3} M (unpublished data). As shown in Figure 2, concentration-relaxation curves for nitroglycerin 10^{-8}–10^{-5} M obtained on arterial rings previously incubated with diethyl maleate 10^{-4} M or 10^{-3} M for 60 min were not significantly different from those from untreated controls.

Discussion

The present results show that the glutathione content of pig coronary arteries was not changed at all following treatment with relatively large doses of nitroglycerin although tolerance (tachyphylaxis) did develop to the relaxant actions of this agent. As prior exposure to nitroglycerin resulted in the development of tolerance under conditions in which glutathione content was unaffected, it is unlikely that depletion of glutathione content is involved in the development of tolerance. It is also unlikely that consumption of glutathione in the vascular smooth muscle is needed for the relaxant response to nitroglycerin. Our results do not, therefore, agree with those of other investigators who proposed that depletion of arterial glutathione content is the cause of tolerance development (Needleman & Johnson, 1973; Needleman *et al.*, 1973). In addition, our findings do not agree with those of Yeates *et al.* (1989) who, using indirect techniques, suggested that glutathione S-transferase activity was inhibited in rabbit aortic strips by nitroglycerin: our direct measurement of glutathione S-transferase activity in pig coronary artery showed no inhibition even with high concentrations of nitroglycerin (10^{-3} M).

As our findings suggested that neither depletion of glutathione content nor loss of glutathione S-transferase activity was involved in the development of tolerance, further experiments were performed using diethyl maleate, a known glutathione depleting agent (Boyland & Chasseaud, 1967; 1968). As expected, diethyl maleate dose-dependently decreased the glutathione content of pig coronary artery, and at 10^{-3} M of diethyl maleate the arterial glutathione content was only just detectable. In contrast, the arterial glutathione S-transferase activity was not changed at all after treatment with diethyl maleate. Under these experimental conditions, nitroglycerin-induced relaxation of potassium-contracted pig coronary artery rings was completely unaffected. This finding supports the hypothesis that glutathione is not involved in the relaxant process or the development of tolerance to nitroglycerin in vascular smooth muscle. The present study, however, does not exclude the idea of Feelisch & Noack (1987) that not glutathione but cysteine may be involved in vascular relaxation and the development of tolerance to nitroglycerin.

In conclusion, prior exposure to high concentrations of nitroglycerin easily produced tachyphylaxis (tolerance) to relaxation in pig isolated coronary artery rings without any change in arterial glutathione content or glutathione S-transferase activity. Furthermore, although arterial glutathione content was almost completely depleted following treatment with diethyl maleate, the relaxant response to nitroglycerin 10^{-8}–10^{-5} M was completely unaffected. Our results suggest that vascular smooth muscle relaxation and tolerance development to nitroglycerin do not involve changes in vascular glutathione content, at least in pig isolated coronary artery.

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Thrombin-activated platelets promote leukotriene B_4 synthesis in polymorphonuclear leucocytes stimulated by physiological agonists

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1 The addition of 2×10^8 human platelets to 8×10^6 polymorphonuclear leucocytes (PMNL) incubated in presence of $2.5\,u\,ml^{-1}$ thrombin and $0.1\,\mu M$ N-formyl-Met-Leu-Phe (FMLP) (or C5a or PAF) led to enhancement of leukotriene B_4 (LTB_4) synthesis by the PMNL (measured by h.p.l.c. as 20-hydroxy- and 20-carboxy-LTB_4) from 4 ± 1 pmol (in absence of platelets) to 26 ± 4 pmol (mean ± s.e.mean, $n = 9$). Platelets and thrombin were both essential for the enhancement of LTB_4 synthesis.

2 Platelets also caused enhancement of LTB_4 synthesis from (30 ± 12 to 134 ± 25 pmol, $n = 6$) when PMNL pretreated with granulocyte-macrophage colony-stimulating factor were used in similar experiments.

3 Enhancement of LTB_4 synthesis was also observed (from 5 ± 1.5 to 26.5 ± 5 pmol, $n = 9$) when the supernatants of thrombin-activated platelet suspensions were added to FMLP-stimulated PMNL.

4 Supernatants of platelet suspensions activated by thrombin in presence of cyclo-oxygenase and 12-lipoxygenase inhibitors led to greater enhancement (from 5 ± 3 to 153.5 ± 27.5 pmol, $n = 3$) of LTB_4 synthesis by FMLP-stimulated PMNL, suggesting that arachidonic acid itself, rather than its metabolites was responsible for the effects of platelets.

5 Addition of arachidonic acid to FMLP-stimulated PMNL at a concentration comparable to that measured in thrombin-activated platelet supernatants ($0.2 \pm 0.025\,\mu M$, $n = 6$) mimicked the effect of platelets or platelet supernatants on LTB_4 synthesis in FMLP-activated PMNL.

6 The present data indicate that under conditions of cell activation by physiological agonists, platelets can significantly increase the formation of the proinflammatory compound LTB_4 in PMNL by providing arachidonic acid. These data lend support to the concept that platelet-PMNL interactions could modulate the inflammatory process.

Keywords: LTB_4 synthesis by neutrophils; platelet-neutrophil interaction; arachidonic acid; granulocyte-macrophage colony-stimulating factor; lipoxygenase and cyclo-oxygenase inhibitors; inflammation

Introduction

Leukotriene B_4 (LTB_4) is a potent proinflammatory compound derived from arachidonic acid through the 5-lipoxygenase pathway; it is synthesized by polymorphonuclear leucocytes (PMNL) stimulated with ionophore A23187 (Borgeat, 1989). LTB_4 is also synthesized by PMNL following receptor-mediated activation by agonists such as the chemotactic peptide N-formyl-Met-Leu-Phe (FMLP), the complement fragment C5a, platelet-activating factor (PAF) or following activation by phagocytosis of particulate stimuli such as zymosan (Claesson *et al.*, 1981; Lin *et al.*, 1982; Clancy *et al.*, 1983; Salari *et al.*, 1985; Borgeat *et al.*, 1988). LTB_4 possesses a number of biological activities *in vitro* and *in vivo*; it is however generally accepted that the most important activity of this eicosanoid lies in its chemotactic and chemokinetic effects on phagocytes, and it is likely that LTB_4 plays an important role in defence mechanisms as well as in inflammatory diseases (Ford-Hutchinson, 1990).

Besides the obvious role of platelets in haemostasis, evidence that cooperation between platelets and PMNL might contribute to promote local inflammation in several pathophysiological situations is accumulating (Weksler, 1988). Furthermore, platelets release arachidonic acid metabolites, PAF, platelet-derived growth factor (PDGF) and platelet factor 4 (PF4) which can affect neutrophil functions such as adhesion, chemotactic activity, secretion and production of superoxide anion (Weksler, 1988). Moreover, activated platelets have been shown to adhere to neutrophils (Jungi *et al.*, 1986; Larsen *et al.*, 1989); such platelet-PMNL interaction might be part of a mechanism leading to the elimination of activated platelets (Larsen *et al.*, 1989), but could also favour the recruitment of platelets at inflammatory sites and enable and facilitate the occurrence of metabolic or functional interactions between the two cell types.

More specifically concerning the metabolism of arachidonic acid, several cellular cooperations between platelets and PMNL have already been demonstrated. LTA_4 released by PMNL is metabolized by platelets into LTC_4 (Maclouf *et al.*, 1989) and lipoxins (Serhan, 1990). It was also demonstrated that PMNL stimulated with the ionophore A23187 in the presence of platelets transform platelet-derived 12S-hydroxy-5,8,10,14-(Z,Z,E,Z)-eicosatetraenoic acid (12-HETE) into 5S,12S-dihydroxy-6,8,10,14(E,Z,E,Z)-eicosatetraenoic acid (5S,12S-DiHETE) (Borgeat *et al.*, 1982; Marcus *et al.*, 1982), and arachidonic acid into LTB_4 (Marcus *et al.*, 1982) via the 5-lipoxygenase pathway. In addition, 12-HETE synthesized in platelets is metabolized to 12S,20-dihydroxy-5,8,10,14(Z,Z,E,Z)-eicosatetraenoic acid (12,20-DiHETE) by unstimulated neutrophils (Marcus *et al.*, 1984). In previous studies we have also shown that incubation of PMNL with high concentration of exogenous arachidonic acid in the presence of platelelets results in increased synthesis of 5-lipoxygenase products. The amplification of 5-lipoxygenase product synthesis in this coincubation system was attributed to the synthesis of 12S-hydroperoxy-5,8,10,14(Z,Z,E,Z)-eicosatetraenoic acid (12-HpETE) by the platelet 12-lipoxygenase (Maclouf *et al.*, 1982). Amplification of LTB_4 synthesis in zymosan-stimulated PMNL was also observed when thrombin-stimulated platelets were present (Del Maschio *et al.*, 1985). These studies clearly indicate that platelets can modulate the synthesis of LTB_4 in

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PMNL and possibly contribute to the development of inflammatory reactions. Further investigations were undertaken to assess the hypothesis that platelet-PMNL interactions leading to increased formation of LTB_4 could also occur under conditions of cell activation by physiological agonists.

In the present study, we show that thrombin-activated platelets strongly increase LTB_4 synthesis by PMNL stimulated with FMLP, C5a or PAF. We have also investigated the mechanism of this effect of platelets and established that arachidonic acid released by platelets is determinant to LTB_4 synthesis by PMNL stimulated with these physiological agonists.

Methods

Materials

5,8,11,14(all *cis*)-eicosatetraenoic acid (arachidonic acid), fatty acid-free human serum albumin (HSA), FMLP, 1-O-hexadecyl-2-acetyl-sn-3-glycerophosphocholine (PAF), prostaglandin B_2 (PGB_2), soybean lipoxidase (type I) and $NaBH_4$ were obtained from Sigma Chemical Company (Saint Louis, MO, U.S.A.). [^{14}C]-arachidonic acid (1.0 Ci mmol^{-1}) was purchased from New England Nuclear (Boston, MA, U.S.A.). Thrombin was from Parke-Davis (Scarborough, Ontario, Canada). Hanks' balanced salt solution (HBSS) and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) were purchased from GIBCO (Burlington, Ontario, Canada). Ficoll-paque was purchased from Pharmacia (Dorval, Quebec, Canada) and solvents were high performance liquid chromatography (h.p.l.c.) grade from Anachemia (Montreal, Quebec, Canada). Biosynthetic recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) was a generous gift from the Genetics Institute (Cambridge, MA, U.S.A.). GM-CSF was diluted in HBSS containing 2.5% foetal calf serum under sterile conditions to a stock concentration of 100 nM which was endotoxin-free as determined by the limulus amoebocyte assay, and stored at −20°C. Recombinant human C5a was a generous gift from Dr Henry Showell (Pfizer Pharmaceuticals, Groton, CT, U.S.A.); it was kept in HBSS at a stock concentration of 30 μM and stored at −20°C. Arachidonic acid was purified by silicic acid chromatography before use. Arachidonic acid and PAF were kept in hexane and chloroform, respectively, and aliquots of stock solutions were evaporated and redissolved in dimethylsulphoxide (DMSO) for addition to cell suspensions; the stock solution of FMLP was prepared in DMSO.

Cell preparation procedures

Venous blood was obtained from healthy donors and collected on citric acid-dextrose anticoagulant. Blood was centrifuged at 200 *g* for 15 min at 20°C. The platelet-rich plasma (PRP) was removed and platelets were isolated as described previously (Lagarde *et al.*, 1980). Briefly, the PRP was acidified to pH 6.4 with 0.15 M citric acid and centrifuged at 900 *g* for 10 min at 20°C in siliconized tubes. Supernatants were removed, and tube walls and platelet pellet surfaces were gently rinsed with Ca^{2+}- and Mg^{2+}-free HBSS containing 10 mM HEPES and buffered to pH 7.4 (HBSS-HEPES). The cells were finally resuspended in the same buffer at 4×10^8 platelets per ml.

PMNL were prepared by sequential dextran sedimentation and centrifugation on Ficoll-paque cushions (Boyum, 1968). Contaminating erythrocytes were eliminated by hypotonic lysis. PMNL were then resuspended in Ca^{2+}- and Mg^{2+}-free HBSS-HEPES buffer at the cell concentration of 16×10^6 per ml, and the viability was evaluated by the trypan blue exclusion test. Cell viability was always greater than 95%.

Platelet-polymorphonuclear leucocyte coincubations

The mixing of equal volumes of the platelet and PMNL preparations resulted in a cell suspension containing 2×10^8 platelets per ml and 8×10^6 PMNL per ml; $CaCl_2$ and $MgCl_2$ were added at final concentrations of 2 mM and 0.5 mM, respectively, and the cells were pre-incubated 5 min at 37°C. The cells were then incubated 10 min at 37°C in presence of various stimuli. In experiments where platelets and PMNL were incubated separately, the platelet and PMNL suspensions were diluted with HBSS-HEPES to obtain final cell concentrations as in the coincubation system. In experiments where GM-CSF was used, PMNL were preincubated with the cytokine at the concentration of 200 pM for 60 min at 37°C in HBSS-HEPES containing 2 mM $CaCl_2$ and 0.5 mM $MgCl_2$ at the cell concentration of 16×10^6 PMNL per ml; coincubation of GM-CSF-primed PMNL with platelets and stimulation were performed as described above. The final concentration of DMSO did not exceed 0.2%.

Incubations were stopped by addition of 0.5 ml of ice cold acetonitrile/methanol (1/1, v/v) containing 12.5 ng each of PGB_2 and 19-hydroxy-PGB_2 as internal standards, and the samples were stored at −20°C. Some coincubation experiments were carried out in the presence of HSA; these incubations were stopped as described above, however the samples were further treated by addition of 3 volumes of acetonitrile (per volume of cell suspension) to achieve complete protein precipitation. The samples were then stored at −20°C until analysed.

Activated platelet supernatants

$CaCl_2$ and $MgCl_2$ were added to platelet suspensions (4×10^8 cells per ml in HBSS-HEPES) to achieve final concentrations of 2 mM and 0.5 mM respectively. The cell suspensions were preincubated 5 min at 37°C and stimulated with 2.5 u ml^{-1} thrombin for 5 min at 37°C. The incubation media were then centrifuged at 2000 *g* for 10 min at 4°C and the supernatants were collected and used immediately for incubation with PMNL suspensions, or were denatured by addition of 2 volumes of acetonitrile and stored at −20°C until analysis. In some experiments activated platelet supernatants were prepared in the presence of 7 μM 5,8,11,14-eicosatetraynoic acid (ETYA) and 0.1 μM tiaprofenic acid, these two inhibitors being added during the preincubation period, i.e. 5 min prior to thrombin activation.

Analysis of 5-lipoxygenase products

The denatured samples were centrifuged at 2000 *g* for 20 min to remove the precipitate material and the supernatants were analyzed without further treatment by reverse phase (RP) h.p.l.c. as described previously (Borgeat & Picard, 1988) with a minor modification of the mobile phase (methanol/acetonitrile/water, 23/23/54, v/v/v, was substituted for acetonitrile/water, 30/70, v/v) to improve the separation of the 20-hydroxy and 20-carboxy derivatives of 5S,12S-DiHETE and LTB_4. The denatured samples initially containing HSA were evaporated down to a volume of ~2 ml at 40°C under a stream of nitrogen prior to RP-h.p.l.c. analysis. Samples were injected onto a Resolve C_{18} Radial Pak cartridge (5 × 100 mm, 5 μm particles) protected by Guard-Pak cartridges (silica and Resolve C_{18}, 5 μm particles) from Waters Millipore. Elution was performed at 1.5 ml min^{-1}. The lipoxygenase products were detected with fixed wavelength u.v. photometers at 229 and 280 nm. Products were identified on the basis of their comigration with synthetic standards and specificity of absorption at either 229 nm or 280 nm. Product quantification was done by comparison of peak heights to calibrated standards of 20-hydroxy-LTB_4, LTB_4 and 15S-hydroxy-5,8,11,13(Z,Z,Z,E)-

eicosatetraenoic acid (15-HETE), after correction for recovery of 19-hydroxy-PGB_2 and PGB_2.

Quantification of arachidonic acid

Arachidonic acid was quantified in supernatants of thrombin-activated platelets by enzymatic and h.p.l.c. procedures. Briefly, the denatured platelet supernatants were centrifuged (2000 *g* for 10 min) to remove the precipitated material and [^{14}C]-arachidonic acid (1 nCi per sample) was added to the supernatants. Arachidonic acid was purified by the RP-h.p.l.c. system described previously (Borgeat & Picard, 1988), using acetic acid instead of phosphoric acid in the mobile phases. The fractions containing arachidonic acid were collected and evaporated at 40°C under a stream of nitrogen. The residues were first dissolved in 50 μl of methanol; then 0.375 ml of 5 mM sodium borate buffer (pH 10) and 100 ng of 8,11,14(all *cis*)-eicosatrienoic acid were added as internal standard to each sample. One thousand units of soybean lipoxidase type I were then added and the reaction mixtures were incubated for 30 min at 20°C to allow the conversion of arachidonic acid and the internal standard into the corresponding 15-hydroperoxide derivatives which were subsequently reduced to 15-HETE and 15S-hydroxy-8,11, 13(Z,Z,E)-eicosatrienoic acid (15-HETrE) by addition of 600 μg of $NaBH_4$ in 1 ml of methanol. After 30 min at 20°C, the reaction was stopped by acidifying the reaction mixtures to pH 5 with acetic acid, and 15-HETE and 15-HETrE were analysed by RP-h.p.l.c. with u.v. detection at 229 nm. Arachidonic acid quantification was performed by comparing peak heights of 15-HETE and 15-HETrE after correction for loss of [^{14}C]-arachidonic acid in the course of the h.p.l.c. purification step. Recovery of arachidonic acid varied between 50 and 70%.

Statistical analysis

Statistical significance was evaluated by Student's *t* test for paired data, unless otherwise indicated.

Results

Effect of platelets on leukotriene B_4 synthesis by polymorphonuclear leucocytes

Figure 1 shows the profiles of lipoxygenase products generated by PMNL suspensions, platelet suspensions and platelet-PMNL mixtures stimulated with 2.5 u ml^{-1} thrombin and 0.1 μM FMLP. The incubation media of PMNL (alone) contained only traces of the metabolite of LTB_4, 20-carboxy-LTB_4 (Figure 1a). Platelets (Figure 1b) responded to stimulation by producing the 12-lipoxygenase product 12-HETE and the cyclo-oxygenase product HHTrE, while 5-lipoxygenase products were not detectable. The absorption peak (280 nm) observed at 16.1–16.2 min elution time on Figure 1b was tentatively identified as a 14,15-dihydroxy-5,8, 10,12-eicosatetraenoic acid (14,15-DiHETE), a product of the 15-lipoxygenase (Maas & Brash, 1983; Wong *et al.*, 1985) on the basis of its u.v. spectrum and comparison with the major 14,15-DiHETE isomer generated by platelets incubated with 15-HpETE (data not shown). Other small absorption peaks at 280 nm, probably isomeric 14,15-DiHETE and 8,15-DiHETE (Maas & Brash, 1983; Wong *et al.*, 1985), were also present in small amounts. FMLP alone did not stimulate the formation of detectable amounts of arachidonic acid metabolites in platelets, such as thrombin in PMNL (data not shown). However, when platelets and PMNL were coincubated and stimulated with thrombin and FMLP (Figure 1c), the formation of 5-lipoxygenase products by the PMNL was strikingly enhanced leading to significant accumulation of 20-hydroxy- and 20-carboxy-LTB_4. The chromatogram also showed the presence of the platelet products 12-HETE, HHTrE as well as

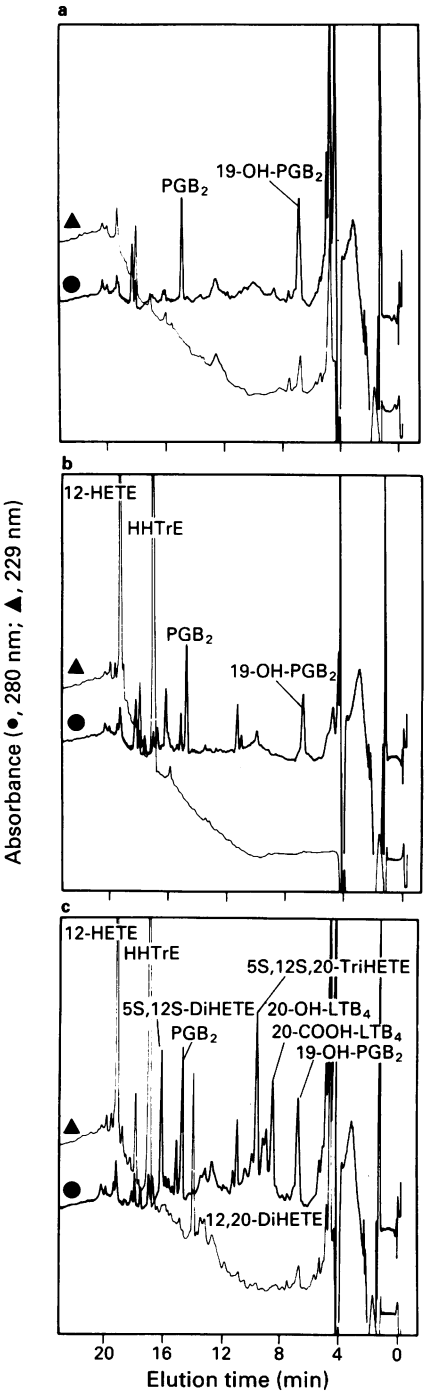

Figure 1 Reverse phase (RP) high performance liquid chromatography (h.p.l.c.) chromatograms of the arachidonic acid metabolites generated by polymorphonuclear leucocytes (PMNL) (a), platelets (b) and a platelet-PMNL mixture (c) incubated 10 min at 37°C with 2.5 u ml^{-1} thrombin and 0.1 μM FMLP. The cells were incubated in 1 ml HBSS with 8 × 10^6 and 200 × 10^6 per ml for PMNL and platelets respectively. The amount of internal standards added was 12.5 ng each of prostaglandin B_2 (PGB_2) and 19-hydroxy-PGB_2. Attenuation settings of the u.v. photometers were 0.01 and 0.025 absorbance units at full scale at 280 and 229 nm, respectively. For abbreviations, see text.

the products of the platelet-PMNL metabolic cooperation, 12,20-DiHETE, 5S,12S-DiHETE which comigrated with the 14,15-DiHETE, and 5S,12S,20-TriHETE. The presence of contaminating material prevented the possibility of detecting LTC_4 synthesis in these experiments.

Figure 2a shows the amount of LTB_4 synthesized either in PMNL alone which were stimulated with 2.5 u ml^{-1} thrombin

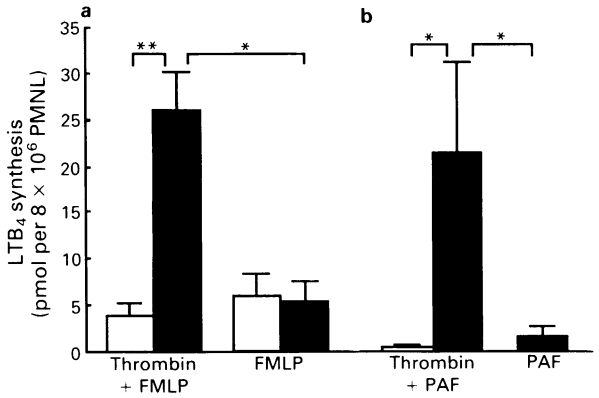

Figure 2 Synthesis of leukotriene B_4 (LTB_4) by human polymorphonuclear leucocytes (PMNL) (open columns) or platelet-PMNL mixtures (solid columns) incubated with 0.1 μM FMLP or FMLP and 2.5 u ml^{-1} thrombin (a) or 0.1 μM PAF or PAF and thrombin (b), for 10 min at 37°C in HBSS. In the cell mixture, the platelet:PMNL ratio was 25:1. LTB_4 was measured by RP-h.p.l.c. and amounts indicated represent the sum of LTB_4, 20-hydroxy-LTB_4 and 20-carboxy-LTB_4. Results are the mean (s.e.mean shown by vertical bars) of 9 experiments (thrombin + FMLP), 3 experiments (FMLP) and 4 experiments (PAF ± thrombin). Incubations were carried out in triplicate in each experiment. ** $P < 0.001$; * $P < 0.05$ for the comparisons indicated.

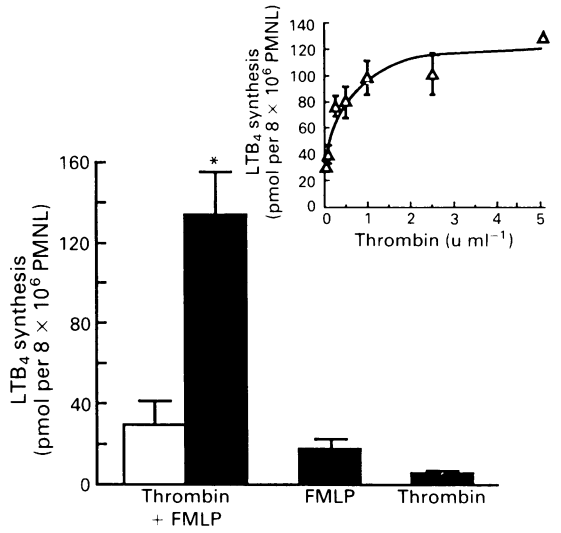

Figure 3 Synthesis of leukotriene B_4 (LTB_4) by GM-CSF-treated polymorphonuclear leucocytes (PMNL) (open column) or platelet-PMNL (GM-CSF-treated) mixtures (solid columns) incubated with 0.1 μM FMLP or 2.5 u ml^{-1} thrombin or both stimuli for 10 min at 37°C in HBSS buffer. PMNL were preincubated 60 min at 37°C in presence of 200 pM GM-CSF. In the cell mixtures the platelet:PMNL ratio was 25:1. LTB_4 was measured by RP-h.p.l.c. and the amounts indicated represent the sum of LTB_4 and its ω-oxidation products. Results are the mean (s.e.mean shown by vertical bars) of 6 experiments (thrombin ± FMLP) or 9 experiments (FMLP); in each experiment, incubations were performed in triplicate. The inset shows the synthesis of LTB_4 by platelet-PMNL (GM-CSF-treated) mixtures incubated with 0.1 μM FMLP and increasing concentrations of thrombin. The results are from one experiment representative of 2 (incubations were done in triplicate and the values are the mean with s.d. shown by vertical bars). * $P < 0.01$ for comparison with the three other experimental conditions.

and/or 0.1 μM FMLP, or in PMNL and platelet mixtures stimulated with either FMLP or both stimuli. Amounts of LTB_4 produced were significantly higher in platelet-PMNL mixtures stimulated with thrombin and FMLP in comparison to PMNL alone stimulated under the same conditions. In nine different experiments, the average increase of LTB_4 (and metabolites) synthesis caused by the coincubation of platelets was 6 fold. The same increment (5 to 6 fold) was observed between platelet-PMNL mixtures stimulated with either FMLP alone or thrombin and FMLP. Furthermore, there was no significant difference in 5-lipoxygenase product synthesis between PMNL alone and platelet-PMNL mixtures stimulated with FMLP only. Leukotriene synthesis was not detectable in unstimulated PMNL or in platelet-PMNL mixtures incubated with thrombin only (data not shown). In these studies the formation of LTB_4 was assessed from the cumulative amounts of LTB_4 itself (often undetectable) and of its metabolites, 20-hydroxy- and 20-carboxy-LTB_4, as LTB_4 is rapidly metabolized by ω-oxidation in PMNL suspensions (Powell, 1984) (Figures 1–3).

Figure 2b shows the results of similar experiments where PAF was used as the PMNL agonist instead of FMLP. The addition of platelets to PMNL incubated in presence of thrombin and PAF also caused a significant increase of LTB_4 synthesis by the PMNL, and the addition of thrombin to platelet-PMNL mixtures incubated in presence of PAF had a similar stimulatory effect.

PMNL were treated with 200 pM GM-CSF for 60 min at 37°C and the effects of FMLP and thrombin, separately or in combination, were investigated on the synthesis of LTB_4 by platelet-PMNL mixtures. Figure 3 clearly shows that stimulation of the cell mixture with both stimuli induced an 8 fold increase in LTB_4 synthesis in comparison to the effect of FMLP alone. Using thrombin alone as a stimulus, leukotriene synthesis was very low. In the same experiments, the addition of platelets to PMNL stimulated with both agonists caused a similar enhancement of LTB_4 synthesis. It is noteworthy that LTB_4 synthesis by platelet and GM-CSF-primed PMNL mixtures stimulated with thrombin and FMLP was increased by 5 to 6 fold in comparison to LTB_4 synthesis observed under the same experimental conditions but using PMNL not previously exposed to GM-CSF (Figures 2 and 3). Comparison of the data presented in Figures 2a and 3 indicated significant differences ($P < 0.01$, established by Student's t test for unpaired samples) in LTB_4 synthesis measured in PMNL or in platelet and PMNL mixtures treated or not with GM-CSF prior to stimulation with thrombin and FMLP.

The Figure 3 inset shows the effect of increasing concentrations of thrombin on LTB_4 synthesis in platelet and GM-CSF-primed PMNL mixtures stimulated with FMLP. Activation of platelets with increasing concentrations of thrombin lead to a concentration-dependent enhancement of LTB_4 synthesis by the PMNL. The effect of thrombin was detectable at 0.1 u ml^{-1} and nearly maximal at 1 u ml^{-1}.

Stimulation of leukotriene B_4 synthesis in polymorphonuclear leucocytes by activated platelet supernatants

Additional experiments were then undertaken to elucidate the mechanism by which activated platelets stimulated LTB_4 synthesis in PMNL. The first series of experiments were aimed at determining whether cell-cell contact was necessary, or alternatively, if active products were released by platelets. Supernatants of thrombin-activated platelet suspensions were obtained as described in Methods. Table 1 shows that the addition of activated platelet supernatants to PMNL stimulated with FMLP or the complement fragment C5a caused significant increase (5 fold or more) of LTB_4 synthesis by the PMNL, indicating that substances released by platelets upon activation by thrombin account for the potentiation of LTB_4 synthesis by the PMNL.

Effect of 12-lipoxygenase and cyclo-oxygenase inhibitors on the stimulatory action of platelets

To test the possibility that products of platelet arachidonic acid metabolism might be involved in the stimulatory effect of activated platelet supernatants, such supernatants were generated in the presence or absence of ETYA and tiaprofenic acid.

Table 1 Leukotriene B_4 (LTB_4) synthesis by polymorphonuclear leucocytes (PMNL) in presence or absence of platelet supernatants

	LTB_4 synthesis (pmol per 8×10^6 PMNL)	
Stimulus	*PMNL*	*PMNL + supernatants*
FMLP	5.1 ± 1.3	26.3 ± 4.9*
C5a	ND	21.2 ± 10.2

PMNL were incubated for 10 min at 37°C in the presence or absence of thrombin-activated platelet supernatants. Values are mean ± s.e.mean of 9 and 4 experiments for FMLP- and C5a-stimulated PMNL, respectively. In each experiment, incubations were carried out in triplicate. Values for LTB_4 synthesis represent the sum of LTB_4 and its ω-oxidation products. * $P < 0.001$ with respect to corresponding value with PMNL. ND, not detectable.

Table 2 shows that the presence of the inhibitors during activation of platelets with thrombin, did not reduce the stimulatory activity of the platelet supernatants on LTB_4 synthesis by the FMLP-stimulated PMNL, but rather, strikingly enhanced this effect. These data clearly indicated that platelet arachidonic acid products were not involved in the stimulatory effect of platelets and suggested that free arachidonic acid, which presumably was increased as the consequence of 12-lipoxygenase and cyclo-oxygenase inhibition by ETYA and tiaprofenic acid, could be involved. Table 3 shows the results of the analysis of 12-HETE, HHTrE and arachidonic acid content of thrombin-activated platelet supernatants; whereas 12-HETE and HHTrE synthesis was almost completely abrogated by the two inhibitors, the amount of arachidonic acid was increased by an average of 17 fold in six experiments, supporting the hypothesis that arachidonic acid released by platelets might account for the stimulatory effect of platelets or platelet supernatants on leukotriene synthesis by PMNL.

Table 2 Effect of inhibitors of platelet arachidonic acid metabolism on leukotriene B_4 (LTB_4) synthesis by activated polymorphonuclear leucocytes (PMNL)

	LTB_4 synthesis (pmol per 8×10^6 PMNL)	
Inhibitors	*PMNL*	*PMNL + supernatants*
—	3.8 ± 1.9	22.5 ± 5.6*
+	5.1 ± 2.9	153 ± 27**†

PMNL were stimulated for 10 min at 37°C in the absence or presence of thrombin-activated platelet supernatants generated in the absence or presence of 0.1 μM tiaprofenic acid and 7 μM ETYA. Values are means ± s.e.mean of 3 experiments. In each experiment, incubations were carried out in triplicate. Values for LTB_4 synthesis represent the sum of LTB_4 and its ω-oxidation products. * $P < 0.05$, ** $P < 0.01$ with respect to PMNL values; † $P < 0.01$ with respect to PMNL + supernatants without inhibitors.

Table 3 Arachidonic acid (AA), 12-HETE and HHTrE in thrombin-activated platelet supernatants

	pmol per 200×10^6 platelets	
Products	*Thrombin*	*Thrombin + inhibitors*
AA	200 ± 25	3500 ± 790
12-HETE	1580 ± 400	<40
HHTrE	1780 ± 160	<20

Platelets were stimulated for 5 min at 37°C with 2.5 u ml^{-1} thrombin and centrifuged at 2000 *g* for 10 min at 4°C in the presence or absence of inhibitors (0.1 μM tiaprofenic acid and 7 μM eicosatetraynoic acid). Results are the mean ± s.e.mean of 6 experiments. In each experiment, incubations were carried out in triplicate. For abbreviations, see text.

Effect of fatty acid-free human serum albumin on the stimulatory action of platelets

In the concentration range of 1 to 5 mg ml^{-1}, HSA dose-dependently inhibited LTB_4 synthesis in platelet-PMNL coincubation in presence of FMLP and thrombin (Figure 4). In similar experimental conditions, HSA did not inhibit FMLP-induced LTB_4 synthesis in PMNL alone (data not shown). These data supported the idea that the potentiation of LTB_4 synthesis could be due to the release of lipophilic substances by activated platelets.

Effect of exogenous arachidonic acid on leukotriene B_4 synthesis in FMLP-stimulated polymorphonuclear leucocytes

Addition of increasing amounts of arachidonic acid to PMNL activated with FMLP led a dose-dependent increase in LTB_4 formation (Figure 5). The effect of arachidonic acid was significant ($P < 0.05$) at 0.1 μM, and at a concentration of 0.2 μM, corresponding to the average concentration measured in activated platelet supernatants generated in absence of ETYA and

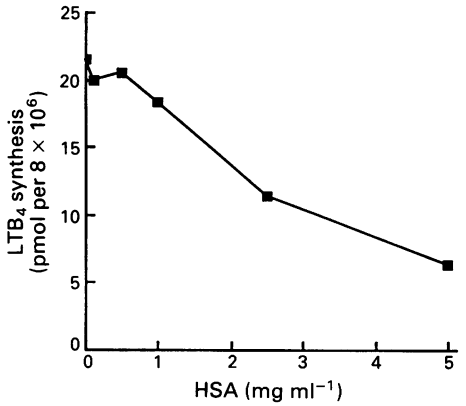

Figure 4 Effect of increasing concentration of fatty acid-free HSA in the incubation media on leukotriene B_4 (LTB_4) synthesis by platelet polymorphonuclear leucocyte (PMNL) mixtures incubated with 0.1 μM FMLP and 2.5 u ml^{-1} thrombin for 10 min at 37°C. The figure shows the results of one experiment representative of 3 (incubations were performed in duplicate).

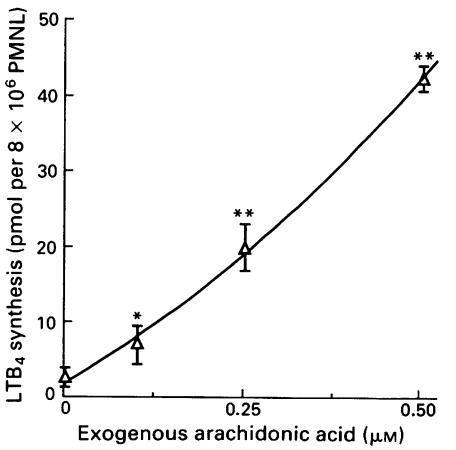

Figure 5 Effect of exogenous arachidonic acid on the synthesis of leukotriene B_4 (LTB_4) (and metabolites) in polymorphonuclear leukocytes (PMNL) incubated 10 min at 37°C in HBSS buffer in presence of 0.1 μM FMLP. LTB_4, 20-hydroxy- and 20-carboxy-LTB_4 were measured by RP-h.p.l.c. Results are the mean (s.e.mean shown by vertical bars) of 3 experiments. Incubations were performed in triplicate in each experiment. * $P < 0.05$; ** $P < 0.005$ compared with arachidonic acid-free incubation.

tiaprofenic acid (Table 3), LTB_4 synthesis was enhanced by ~7 fold.

Discussion

The data presented in this paper clearly show that the coincubation of human PMNL stimulated by soluble physiological agonists, with thrombin-activated platelets results in a significant enhancement of LTB_4 synthesis by the PMNL, in comparison to LTB_4 synthesis by the soluble agonist-stimulated PMNL alone. Enhancement by platelets of LTB_4 synthesis required that both cell types be activated, as in the absence of FMLP, leukotriene synthesis is undetectable (data not shown), and in the absence of thrombin, leukotriene synthesis is comparable to that observed in PMNL alone (Figure 2a). This observation that thrombin is essential for enhancement of LTB_4 synthesis in platelet-PMNL coincubations appears to contradict recent reports that stimulation of platelet-PMNL mixtures by PMNL agonists results in platelet activation (aggregation, 5-hydroxytryptamine release, and synthesis of thromboxane B_2 by platelets) (Del Maschio *et al.*, 1990; Ferrer-Lopez *et al.*, 1990). Presumably, some platelet activation by agonist-stimulated PMNL also occurred in our studies; however, the presence of platelets in activated PMNL suspensions did not lead to significant increase of LTB_4 synthesis (Figure 2) as compared to activated PMNL alone; it is likely that the platelet activation induced by agonist-treated PMNL was of much lower intensity than that achieved by thrombin. In agreement with this hypothesis, platelets incubated in presence of FMLP-stimulated PMNL produced trace amounts of the platelet products 12-HETE and HHTrE (data not shown), while thrombin induced a stronger activation of arachidonic acid metabolism in platelets resulting in the formation of substantial amounts of the two products (Figure 1b).

In addition to increased LTB_4 synthesis, the coincubation of platelets and PMNL resulted in the formation of products derived from metabolic cooperation between the two cell types. Figure 1 shows that activated platelet and PMNL mixtures produced 5S,12S-DiHETE, which is a product of the double dioxygenation of arachidonic acid by the 5- and 12-lipoxygenases (Borgeat *et al.*, 1982; Marcus *et al.*, 1982). The product of the ω-oxidation of the 5S,12S-DiHETE in the PMNL, the 5S,12S,20-TriHETE (Lindgren *et al.*, 1981), was always formed in substantial amounts, while the 12-HETE metabolite, 12,20-DiHETE (Marcus *et al.*, 1984) was also present but in smaller amounts. The mechanism of formation of these three products of platelet-PMNL interaction have been discussed previously (Lindgren *et al.*, 1981; Borgeat *et al.*, 1982; Marcus *et al.*, 1987). The h.p.l.c. system used for the separation of lipoxygenase products in this study enabled the resolution of LTB_4 and 5S,12S-DiHETE (as well as their ω-oxidation products). Furthermore, the use of a high resolution (1.2 nm) photodiode array u.v. detector (Waters Millipore model 994) supported the tentative identification of the compounds by the assessment of small differences in their u.v. spectra (data not shown) reflecting differences in the geometry of the conjugated triene units. The formation of lipoxins, described recently in similar, but not identical experimental conditions (a smaller number of cells was used in the present study) (Serhan & Sheppard, 1990), was not detectable in our studies even using GM-CSF-treated PMNL and monitoring h.p.l.c. effluents at 302 nm for optimal detection.

The effect of thrombin-activated platelets on LTB_4 synthesis was also investigated with PMNL primed by the haematopoietic growth factor, GM-CSF. GM-CSF-treated or 'primed' PMNL show increased functional responses to a second stimulation, such as enhanced superoxide anion formation and enzyme release in response to FMLP (Weisbart *et al.*, 1987; Kaufman *et al.*, 1989), increased phagocytosis (Lopez *et al.*, 1986) and also increased leukotriene synthesis in response to soluble agonists (FMLP, C5a, PAF) and the ionophore A23187 (Silberstein *et al.*, 1986; Dahinden *et al.*, 1988; Dipersio *et al.*, 1988). In the present study, GM-CSF-priming of PMNL led to the expected increase of leukotriene synthesis in response to FMLP (Figures 2a and 3). Moreover the coincubation of the GM-CSF-primed and FMLP-activated PMNL with thrombin-activated platelets led to a further enhancement of LTB_4 synthesis in comparison to the amount of LTB_4 generated in absence of platelets or by platelet-PMNL mixtures stimulated by FMLP only (from 30 to 134 pmol per 8×10^6 PMNL). These results indicate that stimulation of LTB_4 synthesis by activated platelets also occurs in primed PMNL and that under these conditions, LTB_4 synthesis reaches levels exceeding those measured in all other experimental conditions tested.

The results of experiments performed using the supernatants of platelet suspensions (Table 1) clearly indicated that cellular contact was not essential to the stimulatory effect of platelets and that substances released in incubation media following thrombin stimulation of platelets accounted for the effect. The magnitude of the amplification of LTB_4 synthesis by the platelet supernatants was similar to that observed in the coincubation system (Figure 2 and Table 1).

The question of the nature of the active product(s) released by platelets was then addressed. Because 12-HpETE was previously identified as the mediator of the stimulatory effects of platelets on LTB_4 synthesis in a different *in vitro* model (Maclouf *et al.*, 1982), and because thrombin-activated platelets release arachidonic acid metabolites of the cyclooxygenase and the 12-lipoxygenase (Smith *et al.*, 1985), the involvement of one or several of these metabolites (or their precursors) was first considered. Experiments with inhibitors of the metabolism of arachidonic acid were performed. Tiaprofenic acid is a potent inhibitor of cyclo-oxygenase (Schror *et al.*, 1982) whereas ETYA is an inhibitor of the 12- and 15-lipoxygenases and of cyclo-oxygenase (Salari *et al.*, 1984). At a concentration of 7 μM and at the cell concentration used, ETYA showed minimal (< 10%) inhibitory activity on the 5-lipoxygenase in PMNL (data not shown). The results of these experiments (Table 2) clearly show that the inhibitors did not prevent the stimulation of LTB_4 synthesis in PMNL by thrombin-activated platelets, but on the contrary, strongly enhanced the effect of platelets. This suggests that platelet arachidonic acid metabolites were not involved, thus raising the possibility that platelet-derived arachidonic acid is utilized by activated PMNL for LTB_4 synthesis and accounts for the stimulatory effect of activated platelets.

Measurements of arachidonic acid, 12-HETE and HHTrE levels in activated platelet supernatants generated in the presence and absence of the inhibitors supported this hypothesis. Significant amounts of arachidonic acid and of the two metabolites were present in activated platelet supernatants, while the inhibitors completely blocked the formation of 12-HETE and HHTrE, causing a dramatic increase in the level of free substrate (Table 3). Further support for the substrate transfer between platelets and PMNL was provided by experiments using fatty acid-free HSA as an arachidonic acid trapping agent in the incubation media. Indeed HSA inhibited LTB_4 synthesis in platelet-PMNL coincubations in presence of FMLP and thrombin (Figure 4). Measurements of free arachidonic acid levels in these experiments confirmed that the protein acted as substrate scavenger because amounts of arachidonic acid measured in incubation media containing HSA were much higher than in the absence of HSA (data not shown), in agreement with previous reports (Broekman *et al.*, 1989; Purdon & Rao, 1989).

Exogenous arachidonic acid, at the concentration measured in activated platelet supernatants, enhanced LTB_4 synthesis in FMLP-stimulated cells to levels equivalent to those obtained with the activated platelet supernatants (Figures 2 and 5). Furthermore, the profiles of products formed (mainly 20-hydroxy- and 20-carboxy-LTB_4) were identical in the two experimental conditions. Therefore, it can be concluded that arachidonic acid released by thrombin-activated platelets

largely accounts for the stimulatory effect on LTB_4 synthesis in the PMNL.

Besides arachidonic acid and its metabolites, thrombin-activated platelets release other substances such as PAF (and its precursor and metabolite, lyso-PAF), PF4 and PDGF which have been shown to activate PMNL (Deuel *et al.*, 1981; 1982; Lin *et al.*, 1982; Borgeat *et al.*, 1988), and it is difficult to rule out that they might contribute to increase LTB_4 synthesis. However, these products could only play a minor role if any, in the model tested, since provision of substrate appears to account for most of the effect of activated platelets on LTB_4 synthesis.

The present finding that thrombin-activated platelets enhance LTB_4 synthesis in activated PMNL by providing substrate is in agreement with the current understanding of the regulation of leukotriene synthesis. It is indeed well accepted that the PMNL 5-lipoxygenase is present in an inactive form in these cells and that cell stimulation results in enhanced activity of the enzyme (Borgeat & Samuelson, 1979; Rouzer & Samuelson, 1987; Miller *et al.*, 1990). It has also been observed that while PMNL stimulation with physiological agonists such as FMLP, C5a and PAF induces the activation of the 5-lipoxygenase and arachidonic acid release resulting in leukotriene synthesis, the concomitant addition of exogenous substrate leads to a striking increase in the formation of LTB_4 and other 5-lipoxygenase products (Clancy *et al.*, 1983; Salari *et al.*, 1985; Haines *et al.*, 1987; Borgeat *et al.*, 1988) clearly indicating that in PMNL activated by physiological agonists, substrate availability is a limiting factor in the production of LTB_4.

Recent studies demonstrated the enhancement of lipoxin and PAF synthesis in PMNL coincubated with activated platelets lending further support to the implication of platelets in inflammatory processes (Serhan & Sheppard, 1990; Coëffier *et al.*, 1990). The present data also suggest that in pathophysiological situations where PMNL and platelets are activated, in particular in conditions where PMNL are exposed to GM-CSF (or other cytokines with similar effects on leukotriene synthesis, such as TNF (Camussi *et al.*, 1989)), platelet-PMNL interactions may play a role in the development of inflammation by modulating the synthesis of another important lipid mediator, LTB_4.

Our data clearly demonstrate that arachidonic acid released by thrombin-activated platelets accounts for their stimulatory effect on LTB_4 formation in PMNL *in vitro*. Although it seems indeed unlikely that efficient substrate transfer could occur between isolated platelets and PMNL in a physiological environment where plasma proteins would rapidly trap arachidonic acid, it is however conceivable that arachidonic acid could be efficiently exchanged, even in the presence of plasma, between adherent cells. In fact, it has been shown previously that thrombin-activated platelets bind to PMNL (and monocytes) (Jungi *et al.*, 1986); more recently a platelet alpha granule membrane protein (PADGEM or GMP-140) which mediates the specific attachment of platelets to PMNL has been identified and characterized (Larsen *et al.*, 1989; Hamburger & McEver, 1990). These findings strongly support the general concept that specific interactions, including metabolic cooperation occur between these cell types. The data presented constitute a significant step towards the assessment of a role of platelets in inflammation, more specifically in the modulation of mediator release by PMNL. Studies are now in progress to assess the effect of adherent (activated) platelets on leukotriene synthesis in PMNL in plasma and whole blood.

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Role of the σ receptor in the inhibition of [^{3}H]-noradrenaline uptake in brain synaptosomes and adrenal chromaffin cells

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1 Rat brain synaptosomes and cultured bovine adrenal chromaffin cells were used to monitor the inhibitory effects of phencyclidine (PCP) and sigma (σ)-receptor ligands on the uptake of [^{3}H]-noradrenaline ([^{3}H]-NA).

2 A Na^+-dependent high affinity uptake was observed in synaptosomes (30°C) and chromaffin cells (37°C) with K_m of 0.22 and 0.56 μM and V_{max} of 2.5 pmol min^{-1} mg^{-1} protein and 0.7 pmol min^{-1} per 10^6 cells, respectively.

3 PCP and haloperidol inhibited the high affinity uptake with IC_{50} of 0.17 and 0.42 μM, respectively in synaptosomes and 0.24 and 0.47 μM, respectively in adrenal chromaffin cells.

4 A close correlation ($r = 0.96$) was established between the ability of various PCP and σ-receptor ligands to inhibit [^{3}H]-NA uptake in both systems: PCP > TCP > haloperidol > 3-(+)-PPP > MK-801 $\geq$ (−)-butaclamol > (+)-SKF-10047 > DTG. Spiperone and opioid receptor ligands were ineffective at 20 μM.

5 These results indicate that the central and peripheral inhibitory effects of PCP and σ-receptor ligands on [^{3}H]-NA uptake involves a receptor (σ_1-like) which is distinct from that (PCP_2) recognized for the inhibition of [^{3}H]-dopamine uptake by PCP.

Keywords: Phencyclidine receptor; sigma receptor; catecholamine reuptake; synaptosomes; chromaffin cells; adrenal medulla

Introduction

Phencyclidine (PCP) originally introduced in 1958 as a dissociative anaesthetic has since become a major drug of abuse due to its ability to elicit hallucinations and feelings of tranquility (for review see Clouet, 1986). PCP also produces psychotic effects such as delusion, depersonalization, dysphoria and maniacal excitement that resemble the symptoms of schizophrenia (Luby *et al.*, 1959; Allen & Young, 1978). A high affinity binding site for PCP resides inside the ion channel linked to the N-methyl-D-aspartate (NMDA) receptor (Foster & Fagg, 1987). The stimulation of the PCP receptor is involved in the blockade of NMDA-evoked depolarizations of cortical and hippocampal neurones (Thompson *et al.*, 1985; Coan & Collingridge, 1987) and the inhibition of the NMDA-evoked release of dopamine and acetylcholine from brain tissue slices (Snell & Johnson, 1986; Jones *et al.*, 1987; Drejer & Honore, 1987; Snell *et al.*, 1987). PCP can also interact with a low affinity binding site, the sigma (σ)-receptor, sensitive to haloperidol and psychotic benzomorphans such as N-allylnormetazocine ((+)-SKF-10047; Su, 1982; Tam & Cook, 1984). The distinction between PCP and σ-receptors has been established by the distinct distribution of both sites in the brain (Goldman *et al.*, 1985; Largent *et al.*, 1986) and the spinal cord (Aanonsen & Seybold, 1988) and the design of selective ligands for the two receptor sites. Thus, a PCP analogue, N-[2-thienyl]cyclohexyl 3,4-piperidine, (TCP; Vignon *et al.*, 1983) and an anticonvulsant, (+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d]cyclohepten-5,10-imine maleate (MK-801; Sircar *et al.*, 1987) were shown to possess a high affinity and selectivity for the PCP receptor while 3-(3-hydroxyphenyl)-N-(1-propyl)piperidine (3-(+)-PPP; Koe *et al.*, 1989) and 1,3-di(2-tolyl) guanidine (DTG; Weber *et al.*, 1986) selectively bound to the σ-receptor.

Both classes of receptors have been subdivided into two subtypes: the PCP_1 and PCP_2 receptors (Vignon *et al.*, 1986; Rothman *et al.*, 1989) and the σ_1 and σ_2-receptors (Bowen *et al.*, 1989; Hellewell & Bowen, 1990; Itzhak & Stein, 1990). Sigma (σ)-receptors are best characterized by their high affinity for benzomorphans such as (+)-SKF-10047 and their sensitivity to haloperidol. The σ_1-receptor subtype displays high affinity for 3-(+)-PPP and a stereoselective preference for (+)-benzomorphans, as well as a higher molecular weight (25–29 kDa) when compared with the σ_2-receptor subtype (18–21 kDa) (Kavanaugh *et al.*, 1989; Walker *et al.*, 1990). The σ_2-receptor demonstrates high affinity for DTG and stereospecificity for (−)-SKF-10047. All PCP and σ-receptors are insensitive to the opiate antagonist, naloxone. One of the important biological effects of PCP that may be involved in some of its psychotic properties is its blockade of catecholamine and serotonin reuptake by nerve terminals (Smith *et al.*, 1977; Bowyer *et al.*, 1984; Vignon & Lazdunski, 1984; Johnson & Snell, 1985). This effect has been ascribed to the specific interaction of the drug with the PCP_2 receptor (Snell *et al.*, 1988; Vignon *et al.*, 1988; Rothman *et al.*, 1989) as opposed to the PCP_1 receptor which is linked to the NMDA receptor complex. As yet, there has been no report to indicate if the inhibition of the uptake of catecholamines involves the stimulation of the σ receptor and if the PCP_2 receptor is identical to or distinct from the σ receptor or one of its subsets.

The adrenal medulla was recently shown to possess both PCP- and σ-like receptors (Wada *et al.*, 1988; Rogers *et al.*, 1989; Rogers & Lemaire, 1990). One site (PCP-like) was more sensitive to TCP while the other site was more sensitive to haloperidol. The putative physiological role of these binding sites has not been elucidated although PCP and the dissociative anaesthetic, ketamine, were shown to inhibit the nicotine-evoked release of catecholamines from perfused bovine adrenal glands (Malave *et al.*, 1983) and isolated adrenal chromaffin cells (Purifoy & Holz, 1984). Adrenomedullary chromaffin cells constitute a good model to study the mechanism of catecholamine reuptake (Kenigsberg & Trifaró, 1980; Role & Perlman, 1983; Banerjee *et al.*, 1987). These cells originate from the neural crest and display the same characteristics as sympathetic neurones, being able to synthesize, capture, store and release catecholamines (Stjärne, 1972). In this study, we have investigated the effect of PCP and σ-receptor ligands on the uptake of [^{3}H]-NA in bovine cultured

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adrenal chromaffin cells and compared their effects with those obtained with rat isolated brain synaptosomes.

Methods

Chromaffin cell preparation

Bovine adrenal glands obtained from a local slaughterhouse were separated from their cortices and perfused *in vitro* for 10 min at 37°C with Ca^{2+}-free, Mg^{2+}-free Locke solution as described by Fenwick *et al.* (1976). Perfusion was continued for 60 min with fresh solution to which 0.05% collagenase (Sigma Chemical Co., St. Louis, U.S.A.) had been added and chromaffin cells were isolated and cultured as described previously (Fenwick *et al.*, 1976). Cells were cultured for one day and then centrifuged at 900 r.p.m. in a Beckman centrifuge for 10 min and resuspended in buffer A (composition, mM: $NaHCO_3$ 16.2, KCl 4.7, NaCl 133, $CaCl_2$ 2.5, KH_2PO_4 1.2, ascorbic acid 1.14 and nialamide 0.0124 and equilibrated at pH 7.4 with O_2:CO_2, 95%:5%) at a concentration of 5×10^6 cells ml^{-1}. Cell viability was 95% or greater by trypan blue exclusion. At this stage, cells were ready to be included in the uptake assay. The properties of [^{3}H]-NA uptake did not vary significantly from day 2 to 7 of culture (Kenigsberg & Trifaró, 1980); therefore, all assays were performed at day 2.

Preparation of rat brain synaptosomes

Male Wistar rats of the same age and weight (250–275 g) were decapitated, their brains weighed and homogenized in a glass-teflon homogenizer with a clearance of 0.025 mm, containing 0.32 M sucrose (40 ml g^{-1} wet weight). During tissue homogenization, the pestle was rotated at 800 r.p.m. with 12 up and down strokes. The homogenate was centrifuged at 1000 *g* at 4°C in a Sorvall SS34 rotor to remove nuclei and cellular debris. The resulting supernatant was centrifuged at 30,000 *g* at 4°C for 30 min. The pellets (P_2) containing crude synaptosomes were used without further purification (Smith *et al.*, 1977). The synaptosomes were resuspended in 0.32 M sucrose to yield a concentration equivalent to 1.3 mg of protein per ml (Lowry *et al.*, 1959).

Uptake studies

The uptake assay was performed by prewarming tubes containing 600 μl of buffer A, 100 μl of fixed (0.1 μM) or increasing concentrations of [^{3}H]-NA, as indicated, and 100 μl of non-labeled σ, PCP or other receptor ligands (10^{-9}–10^{-4} M) for 5 min before the addition of 200 μl aliquot of either synaptosomes (0.26 mg protein) or chromaffin cells (10^6 cells). This mixture was vortexed and incubated for 10 min (or at the indicated time) at either 0°C (control) or 30°C or 37°C as indicated. Control experiments were also performed in the absence of Na^+ by replacing NaCl and $NaHCO_3$ (buffer A) with equimolar LiCl and $LiHCO_3$, respectively. Samples were then placed on ice for 10 min before filtration by reduced pressure through Whatman GF/B filter circles (Snell *et al.*, 1988). Filters were subsequently washed with 4×3 ml of ice-cold buffer A, transferred to vials containing 8 ml of ACS scintillation cocktail (Amersham) and allowed to equilibrate overnight. Radioactivity was measured in a Beckman LS 7800 Beta counter at 37% efficiency. The total uptake at a given time point or concentration was determined by correction, subtracting the 0°C uptake (or the uptake in Na^+-free medium) from that obtained at 30°C or 37°C. The complex kinetics, composed of saturable and non-saturable components were analyzed by the method of Jaques *et al.* (1984). In the analysis of the data, the non-linear curve-fitting programme, BDATA (EMF Softwares, Knoxville, TN., U.S.A.) was used to determine the values of the K_m and V_{max} of the high affinity uptake. The concentration of PCP and σ-receptor ligands that produced 50% inhibition of [^{3}H]-NA uptake (IC_{50}) were obtained from log-logit plots. Values are means with s.e. of three separate preparations performed in duplicate.

Materials

[^{3}H]-NA (43.7 Ci $mmol^{-1}$) was obtained from New England Nuclear, Boston, MA, U.S.A. PCP and TCP were obtained from Dr H. Avdovich, Health and Welfare, Ottawa, ONT, Canada. Metaphit, (−)-butaclamol, (+)-butaclamol and 3-(+)-PPP were purchased from Research Biochemicals, Natick, MA, U.S.A. Haloperidol, nialamide, dextromethorphan and spiperone were purchased from Sigma Chemical Co., St. Louis, MO., U.S.A. BMY-14802 (α-(4-fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazine butanol) was obtained from Bristol Myers Co., Wallingford, CT., U.S.A. Rimcazole (BW234V: *cis*-9[3,5-dimethyl-1-piperazimyl)propyl]carbazole dihydrochloride) was donated by R.M. Ferris, Burroughs Wellcome, Research Triangle Park, NC., U.S.A. DTG was generously donated by Dr E. Weber, Portland, OR., U.S.A. (+)-SKF-10047 ((+)-N-allynormetazocine) and (−)-SKF-10047 were obtained from Natl. Inst. of Drug Abuse, Baltimore, MD., U.S.A. DAMGOL ([D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin) and DSLET ([D-Ser^2, Leu^5] enkephalinyl-Thr) were supplied by Penninsula Laboratories, CA., U.S.A. U-69593 ((5α, 7α, 8β)-(+)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec8yl)benzeneacetamide) was obtained from the Upjohn Co., Kalamazoo, MI., U.S.A. AP-5 (D-(−)-2-amino-7-phosphonoheptanoic acid) was purchased from Tocris Neuramin, Buckhurst Hill, Essex, U.K.

Results

Kinetics of [^{3}H]-noradrenaline uptake

Incubation of rat brain synaptosomes or bovine isolated adrenal chromaffin cells in the presence of [^{3}H]-NA (0.1 μM) at 30°C or 37°C induced a time- and temperature-dependent uptake of [^{3}H]-NA in both preparations (Figure 1). At 37°C, the synaptosomal uptake rapidly reached a maximum value (15 min) and decreased thereafter, while the chromaffin cell uptake was directly proportional to the time of incubation, up to 45 min. At 30°C, the linearity of the initial uptake lasted longer in both preparations reaching 15 min in synaptosomes and exceeding 60 min in chromaffin cells. The time of incubation was set the same (10 min) for the subsequent assays with both preparations. The linearity of the kinetics of uptake was preserved by setting the temperature at 37°C and 30°C for chromaffin cells and synaptosomes, respectively. Under these conditions, the initial rates of [^{3}H]-NA uptake were 0.65 pmol min^{-1} mg^{-1} synaptosomal protein and 0.08 pmol min^{-1} per 10^6 chromaffin cells.

Cultured adrenal chromaffin cells and isolated synaptosomes were incubated for 10 min in presence of increasing concentrations of [^{3}H]-NA at 37°C and 30°C, respectively. Both preparations displayed high and low affinity uptake processes. The high affinity uptake was Na^+-dependent and possessed the following characteristics: K_m of 0.56 and 0.22 μM for chromaffin cells and synaptosomes, respectively, and V_{max} of 0.7 pmol min^{-1} per 10^6 chromaffin cells and 2.5 pmol min^{-1} mg^{-1} synaptosomal protein. The low affinity uptake was not characterized. Incubation in the absence of Na^+ resulted in complete abolition of the high affinity uptake process without affecting the low affinity uptake. All experiments were then performed at a concentration (0.1 μM) at which [^{3}H]-NA uptake occurred through the Na^+-dependent high affinity site.

Inhibition of [^{3}H]-noradrenaline uptake by PCP and sigma-receptor ligands

The uptake of [^{3}H]-NA was examined in the presence of increasing concentrations of PCP and σ-receptor ligands (Figure 2, Table 1). PCP and haloperidol caused a dose-

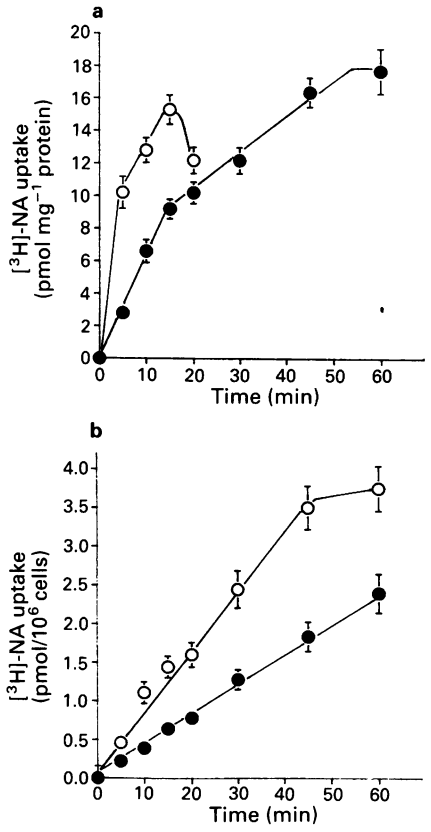

Figure 1 Time- and temperature-dependence of apparent [^{3}H]-noradrenaline ([^{3}H]-NA) uptake into rat brain synaptosomes (a) and bovine cultured adrenal chromaffin cells (b). Synaptosomes and chromaffin cells were incubated at 30°C (●) or 37°C (○) in the presence of [^{3}H]-NA (0.1 μM). Uptake was terminated at various times as described in Methods. Each point represents mean uptake of three sets of duplicates; s.e. shown by vertical bars.

dependent inhibition of [^{3}H]-NA uptake with total inhibition occurring at a concentration of 10–100 μM. The concentration of PCP or haloperidol producing 50% inhibition was quite similar in both systems for PCP (0.17 and 0.24 μM) and for haloperidol (0.42 and 0.47 μM). In order to characterize better the receptor involved in the inhibition of catecholamine uptake, the inhibitory potencies of various specific ligands for PCP and σ-receptors were compared in both uptake systems (Table 1). Among the various PCP receptor ligands tested, PCP and TCP were the most potent (IC_{50} of 0.17–0.42 μM) while MK-801, a specific ligand for the PCP_1 receptor, was less active (IC_{50}: 1.92 and 4.90 μM in chromaffin cells and synaptosomes respectively). The σ-receptor ligands, haloperidol, 3-(+)-PPP, dextromethorphan and rimcazole displayed intermediate potency between PCP and MK-801 with IC_{50} ranging between 0.42 and 4.03 μM. However, other selective σ-receptor ligands such as (+)-SKF-10047, BMY-14802 and DTG were less potent (IC_{50} range of 7.24 to 29.7 μM). The σ-receptor ligand, (+)-SKF-10047, displayed stereoselectivity for the inhibition of [^{3}H]-NA uptake, the (+)-isomer being approximately 3 times more potent than the (−)-isomer in both systems. However, the stereoselective preference for (−)-butaclamol was less evident (Table 1). The D_2 receptor ligand, spiperone, the selective opioid agonists, DAMGOL (μ), DSLET (δ) and U-69593 (κ) and the NMDA receptor antagonist, AP-5 were ineffective at 20 μM.

Table 1 Inhibition of [^{3}H]-noradrenaline ([^{3}H]-NA) uptake by phencyclidine (PCP) and σ-receptor ligands in rat brain synaptosomes and bovine cultured adrenal chromaffin cells

Drug	*Synaptosomes* IC_{50} (μM)[a]	*Chromaffin cells* IC_{50} (μM)[a]
Desmethylimipramine	0.03 ± 0.009	0.01 ± 0.002
PCP ligands		
PCP	0.17 ± 0.09	0.24 ± 0.07
TCP	0.34 ± 0.08	0.42 ± 0.08
Metaphit	0.75 ± 0.11	0.68 ± 0.12
MK-801	4.90 ± 1.34	1.92 ± 0.18
Sigma ligands		
Haloperidol	0.42 ± 0.11	0.47 ± 0.09
3-(+)-PPP	0.82 ± 0.07	0.50 ± 0.08
Dextromethorphan	1.73 ± 0.31	0.51 ± 0.02
Rimcazole	1.96 ± 0.78	4.03 ± 0.28
(−)-Butaclamol	4.06 ± 0.89	5.11 ± 0.12
(+)-Butaclamol	6.06 ± 0.97	8.25 ± 0.15
(+)-SKF-10047	7.24 ± 1.45	14.0 ± 2.87
BMY-14802	9.45 ± 1.16	10.5 ± 2.45
(−)-SKF-10047	20.5 ± 2.46	38.3 ± 6.04
DTG	29.7 ± 2.37	20.0 ± 4.24

[a] The IC_{50} of spiperone (D_2), DAMGOL (μ), DSLET (δ), U-69593 (κ), and AP-5 (NMDA) were ineffective at 20 μM in both systems.
For abbreviations, see text.

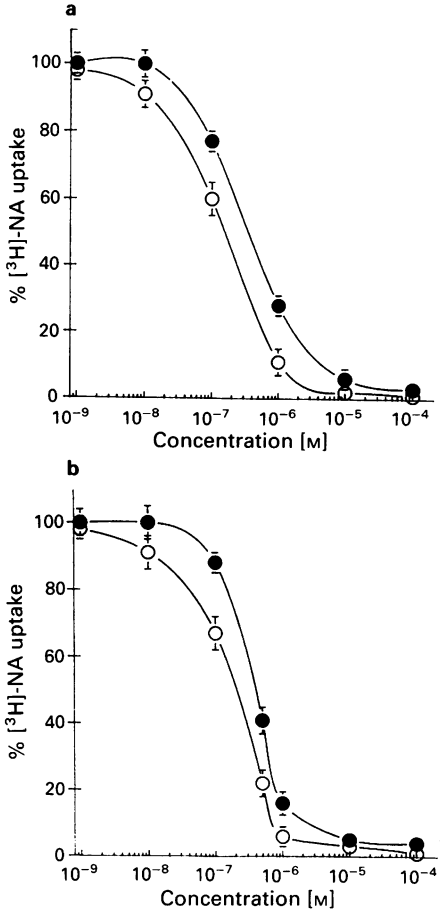

Figure 2 Effects of phencyclidine (PCP) (○) and haloperidol (●) on the uptake of [^{3}H]-noradrenaline ([^{3}H]-NA) into rat brain synaptosomes (a) and bovine cultured adrenal chromaffin cells (b). Synaptosomes and chromaffin cells were incubated at 30°C and 37°C respectively, for 10 min in presence of [^{3}H]-NA (0.1 μM) and increasing concentrations of PCP or haloperidol. Uptake was terminated as described in Methods. Each point represents mean uptake of three sets of duplicates; s.e. shown by vertical bars.

Discussion

The present study indicates that both σ- and PCP-receptor ligands inhibit the uptake of [^{3}H]-NA in rat brain synaptosomes and bovine cultured adrenal chromaffin cells. A

close correlation ($r = 0.96$) exists between the order of potency of these drugs in inhibiting the uptake of [^{3}H]-NA in the two systems (Figure 3), suggesting that the mechanism that mediates this effect is probably the same in the brain and peripheral tissues. The uptake systems for [^{3}H]-NA in crude synaptosomal preparations (for a review see Iversen, 1975) and isolated adrenal chromaffin cells (Kenigsberg & Trifaró, 1980; Role & Perlman, 1983; Jaques *et al.*, 1984; Banerjee *et al.*, 1987) have been described. A high affinity uptake system exists in both tissues. This uptake is temperature- and Na^+-dependent, sensitive to ouabain, cocaine and desmethylimipramine and is saturable and stereospecific. A temperature-sensitive low affinity uptake was also described as Na^+-independent, cocaine-insensitive, non-saturable and not stereospecific. A physiological role for the low affinity uptake site in normal neurotransmission is less probable, although it appears to play an important role in the pharmacological responses to sympathomimetics (Hoffman & Lefkowitz, 1990). Interestingly, the inhibitory effects of PCP and σ-receptor ligands were related to the high affinity uptake (Table 1).

PCP and σ-receptor ligands have multiple binding sites in membrane preparations of rat brain (Quirion *et al.*, 1987; Bowen *et al.*, 1989; Haring *et al.*, 1990; Itzhak & Stein, 1990). The high affinity binding site for PCP is located inside the NMDA receptor-linked ion channel (Foster & Fagg, 1987). A lower affinity PCP binding site was also characterized by use of the prototypic drug, N-[1-(2-benzo (b) thiophenyl) cyclohexyl] piperidine (BTCP), an analogue of PCP, the binding of which was Na^+-dependent. There was also a good correlation between the affinity of compounds to displace the binding of [^{3}H]-BTCP from the PCP_2 site and their potency to inhibit [^{3}H]-dopamine uptake (Vignon *et al.*, 1988). The σ receptor is less well defined but, in the brain, its localization is distinct from that of the PCP_1 binding site (Largent *et al.*, 1986) and it can also be subdivided into two receptor subtypes (Bowen *et al.*, 1989; Hellewell & Bowen, 1990; Itzhak & Stein, 1990).

PCP and σ-like receptors in membrane preparations of bovine adrenal medulla have also been observed (Rogers *et al.*, 1989; Rogers & Lemaire, 1990). It appears that the PCP_1 receptor (NMDA linked) for which MK-801 possesses nanomolar affinity is not present in the adrenal medulla. The adrenomedullary [^{3}H]-TCP binding is sensitive to haloperidol and its pharmacological profile is distinct from both PCP_1 and PCP_2 receptors but rather resembles that of the σ_1-receptor (Rogers & Lemaire, 1990). Yet, the order of potency of PCP and σ-receptor ligands in the inhibition of [^{3}H]-NA uptake in brain synaptosomes and isolated adrenal chromaffin cells is similar ($r = 0.96$; Figure 3), indicating that the receptor(s) involved must possess the same characteristics. This order of potency (PCP > TCP > 3-(+)-PPP > MK-801 > (+)-SKF-10047) also corresponds to the rank order of potency for the potentiation of the contractile response of NA in the rat tail artery (TCP > 3-(+)-PPP > MK-801(+)-SKF-10047) (Massamiri & Piper Duckles, 1989), but not to the order of potency observed for the inhibition of [^{3}H]-BTCP binding to the PCP_2 site, and the blockade of [^{3}H]-dopamine uptake in striatal synaptosomes (Vignon *et al.*, 1988). Since the adrenal medulla contains no PCP_1 binding site, it may prove to be a useful tissue in which to examine the nature of the receptor(s) involved in the inhibition of [^{3}H]-NA uptake by PCP and σ-receptor ligands.

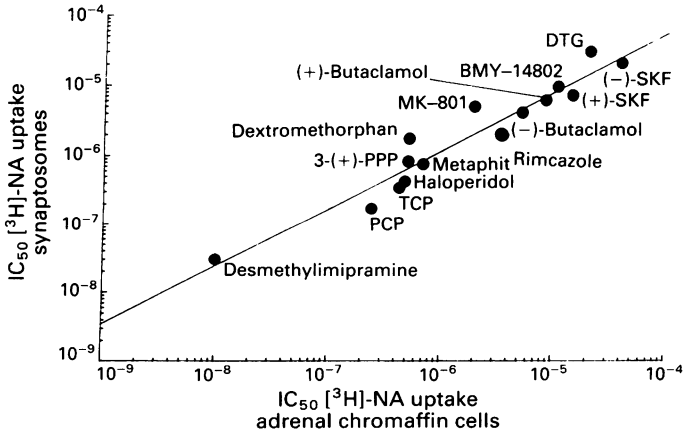

Figure 3 Correlation between the potencies of various phencyclidine (PCP) and σ-receptor ligands in inhibiting the uptake of [^{3}H]-noradrenaline ([^{3}H]-NA) in rat brain synaptosomes and bovine cultured chromaffin cells. The values were taken from Table 1 ($r = 0.96$; (+)- or (−)-SKF = (+)- or (−)-SKF-10047). For other abbreviations, see text.

The brain PCP_2 receptor, responsible for the inhibition of [^{3}H]-dopamine uptake, has been well characterized by the use of the PCP analogue, BTCP, which selectively binds to this receptor and potently inhibits the uptake of [^{3}H]-dopamine (IC_{50}: 13.9 nM; Vignon *et al.*, 1988). However, BTCP appeared to be a relatively poor inhibitor of [^{3}H]-NA uptake (IC_{50} > 10 μM) indicating that this latter effect of PCP-like compounds may involve a distinct receptor. The possible involvement of a distinct receptor was also supported by the relative high potency of MK-801 in the inhibition of [^{3}H]-NA uptake as compared with its inefficiency in inhibiting [^{3}H]-dopamine uptake (Snell & Johnson, 1988; Table 1). In addition, the drug TCP, a potent inhibitor of [^{3}H]-NA uptake in both rat brain synaptosomes and bovine adrenal chromaffin cells (Table 1), was a poor inhibitor of [^{3}H]-BTCP binding and [^{3}H]-dopamine uptake (Vignon *et al.*, 1988) and the displacement of [^{3}H]-TCP binding required high concentrations of BTCP (Rothman *et al.*, 1989).

In the adrenal medulla, [^{3}H]-TCP displayed one high affinity binding component that was potently displaced by haloperidol (K_i: 19 nM) and stereoselectively inhibited by (+)-SKF-10047 (Rogers & Lemaire, 1990), a characteristic of the σ_1-receptor subtype (Walker *et al.*, 1990). Interestingly, the σ-receptor ligands, haloperidol and 3-(+)-PPP were also potent inhibitors of [^{3}H]-NA uptake in rat brain synaptosomes and adrenal chromaffin cells (Table 1). DTG has been shown to be a potent ligand for both σ_1- and σ_2-receptors (Walker *et al.*, 1990), but the effects of GTP on the competition of [^{3}H]-3-(+)-PPP binding by DTG was negligible (Beart *et al.*, 1989), indicating that it may be acting as an antagonist on the 3-(+)-PPP binding site (σ_1; Walker *et al.*, 1990). We did not find any antagonistic activity for DTG in the [^{3}H]-NA uptake assays against PCP or 3-(+)-PPP (data not shown), however, its weak agonist activity (Table 1) may reflect that it is not acting as a pure agonist.

The stereoselective inhibition of [^{3}H]-NA uptake by (+)-SKF-10047 rather than by (−)-SKF-10047 indicates that the receptor involved may be the σ_1 subtype (Hellewell & Bowen, 1990). On the other hand, the similarity between the molecular weights of the σ_1-receptor (25–29 kDa; Kavanaugh *et al.*, 1989; Walker *et al.*, 1990) and that of the PCP_2 receptor (33 kDa; Haring *et al.*, 1987) but not that of the σ_2 (18–21 kDa; Hellewell & Bowen, 1990) or the PCP_1 receptor (90 kDa; Haring *et al.*, 1987) may indicate that the receptors involved in the blockade of dopamine uptake (PCP_2) and NA uptake (σ_1) have a structure that is quite similar. Finally, the NA carrier on which PCP exerts a competitive action (Smith *et al.*, 1977; Schömig *et al.*, 1988) also appears to have a molecular weight (69 kDa; Pacholczyk *et al.*, 1991) that is distinct from those of the PCP_2 and σ_1-receptors.

A clear distinction between all those receptor subtypes will necessitate their isolation and complete structural identification but our results indicate that the selective inhibition of [^{3}H]-NA uptake by PCP and σ-like compounds in the brain and periphery probably involves a receptor site that is distinct from that for the inhibition of [^{3}H]-dopamine uptake (PCP_2 receptor) and which possesses similarities with the σ_1-receptor subtype.

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Inhibitory effects of guanosine 3′:5′-cyclic monophosphate on the synthesis of dopamine in the rat kidney

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1 In the present study the effects of M&B 22,948, a guanosine 3′:5′-cyclic monophosphate (cyclic GMP) selective phosphodiesterase inhibitor and of 8-bromo cyclic GMP were examined on the synthesis of dopamine from L-3,4-dihydroxyphenylalanine (L-DOPA) in rat cortical slices and in whole kidney homogenates. The deamination of newly-formed dopamine into 3,4-dihydroxyphenylacetic acid (DOPAC) was also studied. The assay of L-DOPA, dopamine, noradrenaline and DOPAC was performed by high performance liquid chromatography (h.p.l.c.) with electrochemical detection.

2 Incubation of renal slices and homogenates of whole kidney with exogenous L-DOPA (0.1–10.0 μM) resulted in a concentration-dependent formation of both dopamine and DOPAC.

3 The addition of M&B 22,948 (10 μM) to the incubation medium resulted in a marked reduction in the accumulation of both newly-formed dopamine and DOPAC in kidney slices; the inhibitory effect of M&B 22,948 on DOPAC formation was greater than that on dopamine. 8-Bromo cyclic GMP (250 μM) produced only a slight decrease in the tissue levels of newly-formed dopamine (5–13% reduction), but was found to decrease significantly (51–68% reduction) the formation of DOPAC in kidney slices. The addition of 8-bromo cyclic GMP plus M&B 22,498 to the incubation medium resulted in similar effects to those described for M&B 22,948 alone.

4 In kidney homogenates, in contrast to results observed in kidney slices, M&B 22,948 (10 μM) and 8-bromo cyclic GMP (250 μM) were found to affect neither the formation of dopamine nor its deamination to DOPAC.

5 In conclusion, the results presented here suggest that cyclic GMP may be involved in the regulation of dopamine synthesis, probably through the control of the entry of L-DOPA into the tubular epithelial cells.

Keywords: Dopamine; kidney; dopamine synthesis; guanosine 3′:5′-cyclic monophosphate; L-DOPA (3,4-dihydroxyphenylalanine); DOPAC (3,4-dihydroxyphenylacetic acid)

Introduction

In recent years, evidence suggesting that dopamine plays an important role in renal tissues in the handling of water and electrolytes have been presented (Lee, 1982). Most of the dopamine responsible for these effects is synthesized intrarenally and has its origin in non-neuronal structures. Tubular epithelial cells, namely those of the proximal convoluted tubules, are endowed with a high aromatic L-amino acid decarboxylase (AAAD) activity and filtered 3,4-dihydroxyphenylalanine (DOPA) is converted to dopamine after being taken up into this cellular compartment (Baines, 1982; Hayashi *et al.*, 1990). It has also been reported that endogenous intrarenal dopamine might act physiologically in the control of renal excretion of sodium as a result of activation of dopamine receptors of the D_1-type located in tubular epithelial cells (Jose *et al.*, 1986; Siragy *et al.*, 1989). On the other hand, dietary sodium appears to be a determining factor for the renal production of dopamine, as shown by the close relationship between the concentrations of urinary sodium and urinary dopamine (Cuche *et al.*, 1972; Alexander *et al.*, 1974; Ball *et al.*, 1978).

More recently, evidence has been presented showing that the renal synthesis of dopamine, performed under *in vitro* experimental conditions, is dependent on the concentration of sodium chloride in the medium (Fernandes & Soares-da-Silva, 1990). The renal synthesis of dopamine has also been shown to be reduced by the α-human atrial natriuretic peptide (α-hANP). M&B 22,948, a specific guanosine 3′:5′-cyclic monophosphate (cyclic GMP) phosphodiesterase inhibitor was found to potentiate this effect (Soares-da-Silva & Fernandes, 1990a). ANPs are potent natriuretic agents, the effects of which in renal tissues appear to be dependent on the activation of the particulate form of guanylate cyclase and result in an increased accumulation of cyclic GMP (Hamet *et al.*, 1986). Recently, it has also been reported that the acute infusion of M&B 22,948 to rats resulted in marked natriuretic effect; furthermore, increases in the urinary excretion of cyclic GMP correlated temporally with the natriuresis (McMahon *et al.*, 1989).

The aim of the present work was to study the effects of 8-bromo cyclic GMP and of the cyclic GMP phosphodiesterase inhibitor M&B 22,948 on the synthesis of dopamine in rat cortical slices and kidney homogenates loaded with exogenous L-DOPA and to seek further evidence on the involvement of cyclic GMP in the renal synthesis of dopamine.

Methods

Male Wistar rats (Biotério do Instituto Gulbenkian de Ciência, Oeiras, Portugal) aged 45–60 days and weighing 200–280 g were used in the experiments. Animals were kept two per cage under controlled environmental conditions (12 h light/dark cycle and room temperature 24°C). Food and tap water were allowed *ad libitum*. The experiments were all carried out during day time. The rats were killed by decapitation under ether anaesthesia and both kidneys removed and rinsed free from blood with saline (0.9% w/v NaCl solution). The kidneys were placed on an ice cold glass plate, the kidney poles removed and renal slices approximately 1.5 mm thick, containing both the cortex and the medulla, and weighing about 50 mg wet weight were prepared with a scalpel. Thereafter, renal slices were preincubated for 60 min in 2 ml warmed (37°C) and gassed (95% O_2 and 5% CO_2) Krebs solution. The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, EDTA 0.4, ascorbic acid 0.57 and glucose 11; 1-α-methyl-*p*-tyrosine (50 μM), tropolone (30 μM) and copper sulphate (10 μM) were also added to the Krebs solution in order to inhibit the enzymes tyrosine hydroxylase and catechol-*O*-

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methyltransferase and inhibit the endogenous inhibitors of dopamine β-hydroxylase, respectively. After preincubation, renal slices were incubated for 15 min in Krebs solution with added L-DOPA (10–100 μM). The preincubation and incubation were carried out in glass test tubes, continuously shaken throughout the experiment. In experiments in which the effects of M&B 22,948 (10 μM), 8-bromo cyclic GMP (250 μM) or both, were tested, the compounds were present during the preincubation and incubation periods. After the incubation, renal slices were collected, washed in ice cold saline, blotted with filter paper, weighed and stored in 2 ml of 0.2 M perchloric acid.

In some experiments kidney homogenates, instead of tissue slices, were used. Whole kidneys were homogenized in a modified Krebs solution with Duall-Kontes homogenizers and kept continuously on ice. A modified Krebs solution was also prepared, which was similar to that described above except that NaCl was reduced to 50 mM; the osmolarity of the medium was kept constant by the addition of 68 mM choline chloride. Aliquots of 1.0 ml of kidney homogenates plus 1.0 ml Krebs solution were placed in glass test tubes incubated for 60 min. Thereafter, L-DOPA (0.1–10.0 μM) or dopamine (0.5–5.0 μM) were added to the medium for a further 15 min. During incubation, kidney homogenates were continuously shaken and gassed (95% O_2 and 5% CO_2) and maintained at constant 37°C. The reaction was stopped by the addition of 250 μl of 2 M perchloric acid and the preparations kept at 4°C for 60 min. The kidney homogenates were then centrifuged (2000 r.p.m., 2 min, 4°C) and aliquots of 1.5 ml of the supernatant used for the assay of L-DOPA, dopamine and DOPAC.

The assay of L-DOPA, dopamine, noradrenaline and DOPAC in renal tissues and kidney homogenates was performed by means of high performance liquid chromatography (h.p.l.c.) with electrochemical detection. Aliquots of 1.5 ml of perchloric acid in which tissues had been kept, or 1.5 ml of the supernatant of kidney homogenates were placed in 5 ml conical-based glass vials with 50 mg alumina and the pH of the samples immediately adjusted to pH 8.6 by the addition of Tris buffer. Mechanical shaking for 10 min was followed by centrifugation and the supernatant discarded. The adsorbed catecholamines were then eluted from the alumina with 200 μl of 0.2 M perchloric acid on Millipore microfilters (MF1); 50 μl of the eluate was injected into a high pressure liquid chromatograph with electrochemical detection. The chromatography system consisted of a pump (Gilson model 302; Gilson Medical Electronics, Villiers le Bel, France) connected to a manometric module (Gilson model 802 C) and a stainless steel 5 μm ODS column (Biophase; Bioanalytical Systems, West Lafayette, Indiana, U.S.A.) of 25 cm length. Samples were injected into the column by means of an automatic sample injector (Gilson model 231) connected to Gilson dilutor (model 401). The detection was carried out electrochemically by means of a thin-layer cell with a glassy carbon working electrode, an Ag/AgCl reference electrode, and an amperometric detector (Gilson model 141). The detector cell was operated at 0.75 V. The current produced was monitored with a Spectra-Physics integrator (model SP4270; Spectra-Physics Darmstadt-Kranichstein, FRG). The mobile phase was a degassed solution of citric acid (0.1 mM), sodium octylsulphate (0.5 mM), sodium acetate (0.1 M), EDTA (0.17 mM), dibutylamine (1 mM) and methanol (10% v/v) and pumped at a rate of 1.0 ml min^{-1}. The pH of the mobile phase was adjusted to pH 3.5 by the addition of perchloric acid. Standard solutions of L-DOPA, noradrenaline, dopamine, DOPAC and dihydroxybenzylamine (internal standard) were injected at different concentrations and peak height increased linearly. The inter assay coefficient of variation was less than 5%. The lower limits for detection of L-DOPA, noradrenaline, dopamine and DOPAC were 1.0, 0.9, 1.4 and 2.5 pmol g^{-1}, respectively.

Mean values ± s.e.mean of n experiments are given. Significance of differences between one control and several experimental groups was evaluated by Tuckey-Kramer method (Sokal & Rohlf, 1981). A P value less than 0.05 was assumed to denote a significant difference.

The protein content of the homogenates (mg of protein per g of tissues) was determined by the method of Lowry *et al.* (1951), with human serum albumin as a standard.

8-Bromo cyclic guanosine 3':5'-monophosphate, 3,4-dihydroxyphenylacetic acid (DOPAC), L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine hydrochloride, 1-α-methyl-*p*-tyrosine, noradrenaline bitartrate and tropolone hydrochloride were purchased from Sigma Chemical Company (St. Louis, Mo, U.S.A.) and M&B 22,948 (2-*o*-propoxyphenyl-8-azapurin-6-one) was kindly donated by May & Baker, Ltd (Dagenham, England).

Results

As shown in Figure 1a, the accumulation of L-DOPA in kidney slices was found to be dependent on the concentration of the amino acid added to the medium; the addition of M&B

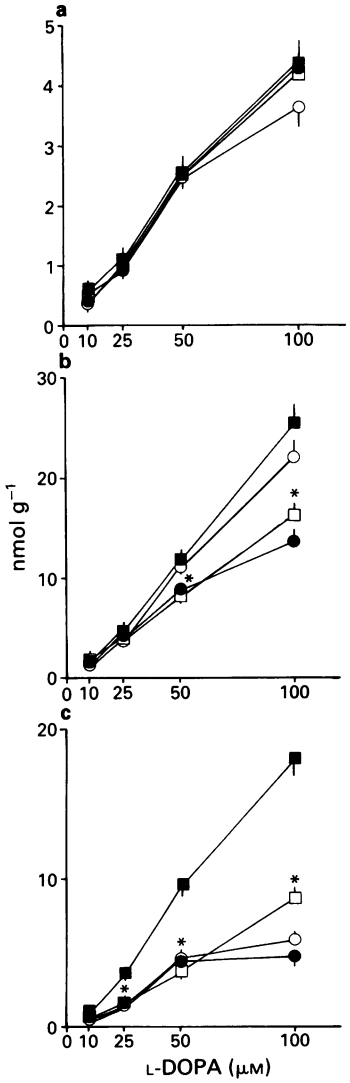

Figure 1 Tissue levels (in nmol g^{-1}) of (a) L-DOPA, (b) dopamine and (c) 3,4-dihydroxyphenylacetic acid (DOPAC) in slices containing both the renal cortex and renal medulla incubated with increasing concentrations of L-DOPA (10–100 μM) for 15 min in control conditions and in the presence of M&B 22,948 (10 μM), 8-bromo cyclic GMP (250 μM) or M&B 22,948 plus 8-bromo cyclic GMP. Each point represents the mean of five to six experiments per group; vertical lines show s.e.mean. Significantly different from corresponding values of control using the Tuckey-Kramer method (* $P < 0.01$). Control, (■); M&B 22,948, (□); 8-bromo cyclic GMP, (○); M&B 22,948 plus 8-bromo cyclic GMP, (●).

Table 1 Effects of M&B 22,948, 8-bromo cyclic GMP and M&B 22,948 plus 8-bromo cyclic GMP on the decarboxylation of added L-DOPA in rat kidney slices

L-DOPA	*Control*	*M&B 22,948*	*8-bromo cyclic GMP*	*M&B 22,948 + 8-bromo cyclic GMP*
10 μM	2.3 ± 0.3	1.4 ± 0.2	1.9 ± 0.2	1.7 ± 0.2
25 μM	8.0 ± 1.3	5.7 ± 0.3	5.3 ± 0.6	5.6 ± 0.6
50 μM	21.2 ± 2.3	13.0 ± 1.5**	15.9 ± 1.9*	13.2 ± 1.0**
100 μM	43.3 ± 6.2	25.0 ± 2.5**	27.9 ± 3.1**	18.5 ± 1.9**

The results are expressed as the algebraic sum of tissue contents of newly-formed dopamine and DOPAC (in nmol g^{-1}). Values are means ± s.e.mean of 6 experiments per group.
Significantly different from corresponding control values using Tuckey-Kramer method (* $P < 0.02$, ** $P < 0.01$).

22,948 (10 μM), 8-bromo cyclic GMP (250 μM) or both to the incubation medium was found not to affect the accumulation of L-DOPA in kidney slices (Figure 1a). Incubation of kidney slices in the presence of increasing concentrations of L-DOPA also resulted in a concentration-dependent accumulation of newly-formed dopamine (Figure 1b). The tissue levels of noradrenaline in kidney slices did not change during the course of these experiments even when 100 μM L-DOPA was used (data not shown). The tissue levels of DOPAC, the deaminated metabolite of dopamine, in kidney slices were also found to be dependent on the concentration of L-DOPA used and reached values as high as 18 nmol g^{-1} when 100 μM L-DOPA was used (Figure 1c). In kidney slices incubated in the absence of exogenous L-DOPA, the dopamine and noradrenaline tissue levels were 0.024 ± 0.006 and 0.590 ± 0.052 nmol g^{-1}, respectively; DOPAC was not detectable under these experimental conditions.

The addition of M&B 22,948 to the incubation medium resulted in a marked reduction in the accumulation of both newly-formed dopamine and DOPAC in kidney slices (Figure 1b and 1c). The inhibitory effect of M&B 22,948 on the formation of dopamine was found to be significant only when tissues were incubated with 50 and 100 μM L-DOPA; the percentage reduction of dopamine tissue levels by M&B 22,948 was about the same when either 50 or 100 μM L-DOPA was used (31 and 36% reduction, respectively). The inhibitory effect of M&B 22,948 on DOPAC formation was greater than that on dopamine; M&B 22,948 reduced DOPAC tissue levels by 55%. With 100 μM L-DOPA in the medium, DOPAC/dopamine tissue ratios were 0.73 ± 0.06 and 0.49 ± 0.05, in the absence and the presence of M&B 22,948 respectively ($P < 0.01$, $n = 6$).

8-Bromo cyclic GMP slightly reduced the tissue levels of newly-formed dopamine in kidney slices (5–13% reduction), but this effect did not attain statistical significance (Figure 1b). By contrast, 8-bromo cyclic GMP was found to reduce significantly the tissue levels of DOPAC in kidney slices loaded with L-DOPA; this effect was observed at all concentrations of the amino acid, although it was more pronounced at 100 μM L-DOPA (68% reduction) than at 50 μM L-DOPA (51% reduction) (Figure 1c). However, as shown in Table 1, the inhibitory effect of 8-bromo cyclic GMP upon the accumulation of the total amount of dopamine formed (as indicated by the algebraic sum of the tissue levels of dopamine and DOPAC) was about the same as that produced by the cyclic GMP phosphodiesterase inhibitor M&B 22,948. The more effective reduction in the formation of DOPAC than dopamine by 8-bromo cyclic GMP also resulted in a significant ($P < 0.01$) decrease of the DOPAC/dopamine tissue ratios. In the presence of 8-bromo cyclic GMP, DOPAC/dopamine tissue ratios were further reduced to 0.24 ± 0.03 ($n = 6$; $P < 0.01$), with 100 μM L-DOPA in the medium.

The addition of 8-bromo cyclic GMP plus M&B 22,498 to the incubation medium resulted in similar effects to those described for M&B 22,948 alone (Figure 1b and 1c; see also Table 1). The combination of 8-bromo cyclic GMP plus M&B 22,498 resulted in a significant reduction in the formation of both dopamine and DOPAC, though it was more effective upon DOPAC formation.

As shown in Figure 2, the synthesis of dopamine and its deamination to DOPAC in kidney homogenates closely depended on the concentration of L-DOPA added to the medium. In contrast to that observed in kidney slices, M&B 22,948 (10 μM) and 8-bromo cyclic GMP (250 μM) were found neither to affect the formation of dopamine nor its deamination to DOPAC in homogenates of the rat kidney.

Incubation of kidney homogenates with exogenous dopamine (0.5, 2.5 and 5.0 μM) was found to result in a concentration-dependent formation of DOPAC (Figure 3). In this set of experiments, as has been found to occur in experiments in which kidney homogenates were incubated with L-DOPA, the addition of M&B 22,948 or 8-bromo cyclic GMP to the incubation medium was found not to affect the formation of DOPAC.

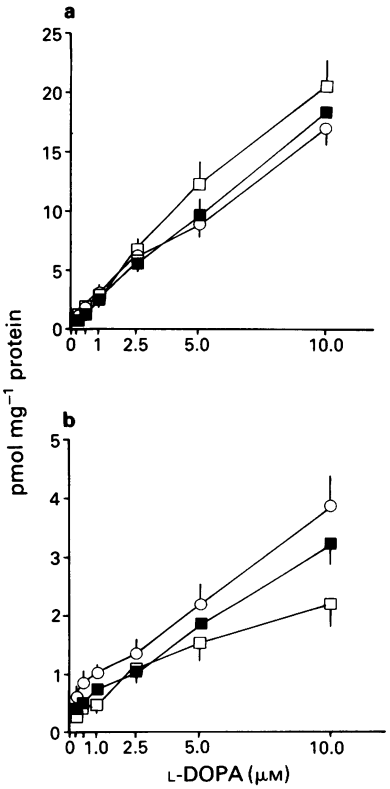

Figure 2 Levels (in pmol mg^{-1} protein) of (a) dopamine and (b) 3,4-dihydroxyphenylacetic acid (DOPAC) in whole kidney homogenates incubated with increasing concentrations of L-DOPA (0.1–10.0 μM) for 15 min in control conditions and in the presence of M&B 22,948 (10 μM) or 8-bromo cyclic GMP (250 μM). Each point represents the mean of five to six experiments per group; vertical lines show s.e.mean. Control (■); M&B 22,948, (□); 8-bromo cyclic GMP, (○).

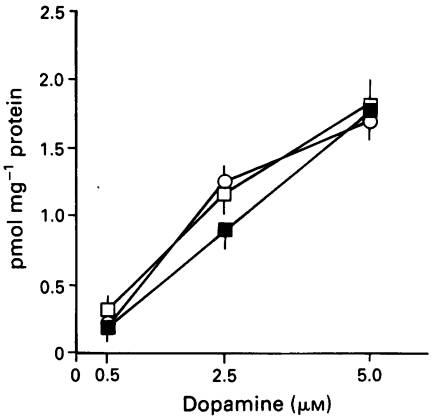

Figure 3 Levels (in pmol mg^{-1} protein) of 3,4-dihydroxyphenylacetic acid (DOPAC) in whole kidney homogenates incubated with increasing concentrations of dopamine (0.5–5.0 μM) for 15 min in control conditions and in the presence of M&B 22,948 (10 μM) or 8-bromo cyclic GMP (250 μM). Each point represents the mean of four experiments per group; vertical lines show s.e.mean. Control, (■); M&B 22,948, (□); 8-bromo cyclic GMP, (○).

Discussion

The results presented here show that both the cyclic GMP phosphodiesterase inhibitor, M&B 22,948, and 8-bromo cyclic GMP effectively reduced the synthesis of dopamine in kidney slices loaded with exogenous L-DOPA. Phosphodiesterases represent the sole pathway in the degradation of cyclic nucleotides; it is, therefore, expected that the effects obtained during the inhibition of this metabolic process might be related to an increased accumulation of the nucleotides (Waldman & Murad, 1987). On the other hand, exogenously applied cyclic GMP or a more lipophilic compound and probably easier penetrating derivative, such as 8-bromo cyclic GMP (Schultz *et al.*, 1979), should be able to mimic such an effect. According to this view, the data given here would suggest that cyclic GMP might be involved in the regulation of the renal synthesis of dopamine.

The results obtained in this study do not provide direct evidence on the processes through which M&B 22,948 and 8-bromo cyclic GMP decrease the synthesis of dopamine in renal tissues. There is, however, evidence to suggest that the inhibitory effects of M&B 22,948 and 8-bromo cyclic GMP on the formation of dopamine might be related to a decrease in both the tubular transport and intracellular availability of L-DOPA. Firstly, neither M&B 22,948 nor 8-bromo cyclic GMP alter the decarboxylation of exogenous L-DOPA when the integrity of the tubular epithelial cells has been disrupted, as it occurs in homogenates of the rat kidney. Secondly, both the tubular transport of L-DOPA and the renal synthesis of dopamine have been found to be highly correlated with the renal delivery of sodium (Lee, 1982; Soares-da-Silva & Fernandes, 1990a,b) and it has been suggested that cyclic GMP is an effective inhibitor of the tubular transport of sodium (Hamet *et al.*, 1986; Zeidel *et al.*, 1987; Lewis *et al.*, 1988; McMahon *et al.*, 1989).

The effects of 8-bromo cyclic GMP upon the synthesis of dopamine, however, did not closely follow those of M&B 22,948. In fact, 8-bromo cyclic GMP reduced the accumulation of the deaminated metabolite of dopamine, DOPAC, more effectively than M&B 22,948. On the other hand, the phosphodiesterase inhibitor was more effective in reducing the accumulation of newly-formed dopamine than 8-bromo cyclic GMP. However, the less pronounced effect of 8-bromo cyclic GMP upon the accumulation of dopamine appears to be the result of a greater inhibitory effect upon the process of deamination of the amine; i.e., the amount of the amine which is not going to be deaminated would be accumulated and would tend to compensate for the inhibitory effect on the formation of the amine. In fact, when the algebraic sum of the tissue levels of newly-formed dopamine and DOPAC is considered, as the result of decarboxylation of the added L-DOPA, it becomes evident that both 8-bromo cyclic GMP and M&B 22,948 produced an effective decrease in the decarboxylation process of the amino acid. It might be hypothesized, therefore, that cyclic GMP not only affects the formation of dopamine, but may also interfere with the disposition of the amine. This seems to agree with the results obtained in this study and would support a hypothetical explanation for the discrepant results obtained with M&B 22,948 and 8-bromo cyclic GMP. However, the results obtained in experiments performed in kidney homogenates suggest that the effects of 8-bromo cyclic GMP on the formation of DOPAC do not appear to be related to a direct inhibitory effect of cyclic GMP upon the enzyme, monoamine oxidase (MAO). Although with less effect than 8-bromo cyclic GMP, M&B 22,948 was also found to decrease more intensely the accumulation of DOPAC in renal tissues than the accumulation of newly-formed dopamine, as indicated by the decrease of the DOPAC/dopamine tissue ratios. This effect, however, as has been found for 8-bromo cyclic GMP, could not be observed in experiments performed in kidney homogenates. Taken together, this would lead to the suggestion that cyclic GMP may also interfere in the process of compartmentalization of dopamine in tubular epithelial cells, by reducing the access of the amine to MAO.

M&B 22,948 was originally thought to be a specific cyclic GMP phosphodiesterase inhibitor. More recently however, it has been shown that increases in adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels may also occur after exposure to M&B 22,948. This effect on cyclic AMP levels depends on the concentration of M&B 22,948 used and appears to present some tissue selectivity (Schoeffter *et al.*, 1987; McMahon *et al.*, 1989). According to Kukovetz *et al.* (1979), M&B 22,948 was found, under *in vitro* experimental conditions, to inhibit the hydrolysis of cyclic GMP more effectively than that of cyclic AMP; the corresponding K_i values were 18 μM for cyclic GMP hydrolysis and 93 μM for cyclic AMP hydrolysis. Thus, it appears that in the present study the effects obtained with M&B 22,948 (10 μM) were most probably due to an increased accumulation of cyclic GMP in renal tissues.

In conclusion, the results presented here suggest that cyclic GMP may be involved in the regulation of dopamine synthesis and disposition of newly-formed dopamine in tubular epithelial cells.

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Identification of a D_1 dopamine receptor, not linked to adenylate cyclase, on lactotroph cells

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1 We studied the lactotroph cells of the rat by both *in vivo* and *in vitro* pharmacological techniques for the presence of D_1-receptors. Both approaches revealed the presence of a D_2-receptor, stimulated by quinpirole (resulting in an inhibition of prolactin secretion) and blocked by domperidone.

2 Administration of fenoldopam, the most selective D_1-receptor agonist currently available, resulted in a dose-dependent decrease of prolactin secretion *in vivo* (after pretreatment with α-methyl-*p*-tyrosine) and *in vitro* (cultured pituitary cells). This increase was dose-dependently blocked by the selective D_1-receptor antagonist, SCH 23390, and although the effect of fenoldopam was less than that obtained by D_2-receptor stimulation, these data suggest that a D_1-receptor also controls prolactin secretion.

3 In order to detect the location of these dopamine receptors, autoradiographic studies were performed by use of [^{3}H]-SCH 23390 and [^{3}H]-spiperone as markers for D_1- and D_2-receptors, respectively. Specific binding sites for [^{3}H]-SCH 23390 were demonstrated. Fenoldopam dose-dependently reduced [^{3}H]-SCH 23390 binding, but had no effect on [^{3}H]-spiperone binding. Immunocytochemical labelling of prolactin cells after incubation with [^{3}H]-SCH 23390 revealed that the granulae and hence, D_1 binding sites were present on the lactotroph cells.

4 Radioligand binding studies performed on membranes from anterior pituitary cells revealed the presence of the D_2-receptor (54 fmol mg^{-1} protein) with a K_d of 0.58 nM for [^{3}H]-spiperone, but failed to detect D_1-receptors.

5 Finally, we studied the effect of dopamine and of fenoldopam on the adenosine 3':5'-cyclic monophosphate (cyclic AMP) content of anterior pituitary cells. Although cyclic AMP increased upon prostacyclin administration, indicating an intact adenylate cyclase system, fenoldopam failed to increase the cyclic AMP production.

6 It is tempting to speculate that fenoldopam reduces prolactin secretion through interaction with a non-cyclase-linked D_1-receptor on the lactotroph cells.

Keywords: Dopamine; D_1-receptors; D_2-receptors; prolactin secretion

Introduction

Some ten years ago Kebabian & Calne (1979) proposed the division of central dopamine receptors into two subtypes, according to biochemical and pharmacological criteria: the D_1-receptors, which are positively coupled to adenylate cyclase, and the D_2-receptors, which are negatively coupled to this enzyme. The main pharmacological characteristics of D_2-receptors are their high affinity for neuroleptics and their lower affinity for dopamine receptor agonists (Seeman & Grigoriadis, 1987). Independently, two subtypes of dopamine receptors were identified in the periphery, which were designated as DA_1- and DA_2-receptors. Their characteristics are very similar, but not identical, to those of the D_1- and D_2-receptors in the central nervous system (Stoof & Kebabian, 1984).

Dopamine has been shown to be an important regulator of prolactin secretion from the anterior pituitary gland (Denef, 1986). Stimulation of this pituitary dopamine receptor results in a small decrease in prolactin secretion. Therefore the hypothesis of a tonic inhibition of prolactin secretion by dopamine has been put forward (Gunnet & Moore, 1988). Blockade of the dopamine receptor by drugs such as domperidone, markedly increases the prolactin release. In rat anterior pituitary homogenates, dopamine inhibits basal adenylate cyclase by approximately 50% (Enjalbert *et al.*, 1986), and both radioligand binding and autoradiographic studies have demonstrated the presence of D_2-binding sites on the prolactin secreting cells (Enjalbert & Bockaert, 1983; Foord *et al.*, 1983; Nikitovich-Winer *et al.*, 1987).

These findings have led to the suggestion that the pituitary dopamine receptor, involved in prolactin secretion, is a D_2-receptor. Moreover, prolactin secretion by lactotroph cells has been currently used as a model to evaluate D_2-receptor activity of dopamine-like drugs.

In contrast to the well established presence of a D_2-receptor, initial studies concerning the presence of a D_1-receptor yielded contradictory results. The selective D_1-receptor antagonist, SCH 23390 (Hyttel, 1983), has been shown to stimulate prolactin secretion in the rat (Apud *et al.*, 1985); however, when [^{3}H]-SCH 23390 was used as an autoradiographic ligand, no specific binding sites in broken cells from the anterior pituitary gland could be demonstrated (Rovescalli *et al.*, 1987). SK&F 38393-A, a relatively selective D_1-receptor agonist, has been reported to either stimulate (Saller & Salama, 1986) or inhibit prolactin secretion (Cocchi *et al.*, 1987). This prompted us to investigate the effects of fenoldopam, the most selective D_1-receptor agonist currently available (Hahn *et al.*, 1982), on prolactin secretion in the conscious rat and from cultured pituitary cells, and to compare these results with those obtained with quinpirole, a selective D_2-receptor agonist (Lefevre-Borg *et al.*, 1987). In this study, it is shown that fenoldopam and quinpirole both lower prolactin secretion, suggesting the presence of both, D_2- and D_1-receptors. Whereas initial attempts to detect D_1-receptors by binding of [^{3}H]-SCH 23390 to broken cell preparations

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were unsuccessful, their presence on intact cultured pituitary cells can be demonstrated by autoradiography.

Methods

In vivo *experiments*

The *in vivo* studies were done in 19 groups of either 6 or 12 normotensive male Wistar Kyoto rats (KUL Proefdierencentrum, Leuven, Belgium), which were equipped with an indwelling silastic catheter, implanted in the right external jugular vein under sodium pentobarbitone anaesthesia (40 mg kg^{-1}, i.p.). The catheters were exteriorized and kept open by daily flushing with a heparinized saline solution.

Rats were housed individually in wire-bottom cages in a light controlled room with a 10:14 h light:dark cycle (lights on at 07 h 00 min), having food and water freely available. All experiments were conducted 4 to 6 days after surgical procedure.

In a preliminary series of experiments saline ($n = 6$), fenoldopam (100 μg kg^{-1}; $n = 6$) or quinpirole (100 μg kg^{-1}; $n = 6$) was administered after an initial blood sampling (0.7 ml). Two additional blood samples were drawn 15 and 40 min after injection of the different drugs.

As the synthesis and release of prolactin are under a predominantly inhibitory influence of hypothalamic dopamine, we also assessed the effects of the different dopamine receptor agonists on α-methyl-*p*-tyrosine (AMPT) treated rats. AMPT, a tyrosine hydroxylase inhibitor, decreases temporarily the endogenous dopamine production at the medio-basal-hypothalamic dopaminergic neurones. This results in a diminished dopaminergic tone at the pituitary gland and a subsequent increase of plasma prolactin (Buydens *et al.*, 1988).

Therefore, in the second series of experiments AMPT (250 mg kg^{-1}, i.p.) was administered, after initial blood sampling. A second blood sample was taken 1 h later, after which either saline ($n = 48$) or the selective dopamine D_1 receptor antagonist, SCH 23390 (100 μg kg^{-1}; $n = 48$) was administered intravenously. A third blood sample, drawn 10 min later, was immediately followed by injection of either saline ($n = 12$), fenoldopam (10, 30, 100 or 300 μg kg^{-1}, $n = 12$ for each dose) or the selective dopamine D_2-receptor agonist, quinpirole (10, 30 or 100 μg kg^{-1}, $n = 12$ for each dose). The doses of fenoldopam and quinpirole used are those producing maximal reductions in renal and mesenteric vascular resistance (Van der Niepen *et al.*, 1988; Dupont *et al.*, 1987). Two additional blood samples were taken 15 and 40 min after injection of these drugs. Blood samples were always immediately centrifuged and plasma was stored at −20°C until assayed for prolactin. Red blood cells were resuspended in heparinized saline (0.25 ml) and reinfused after the following blood sampling to minimize the effect of blood loss.

In vitro *experiments*

For each experiment, total pituitary cell populations were prepared from anterior pituitaries of 20 female adult nulliparous Wistar rats as described by Velkeniers *et al.* (1988). The cells were subsequently grown in 24-well dishes (Falcon) at concentrations of 5×10^4 cells in 1 ml of culture medium, and maintained at 37°C in 5% CO_2 humidified atmosphere. Culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with $NaHCO_3$ 1 g l^{-1} HEPES (4-{2-hydroxyethyl} piperazin-N-1-ethanesulphonic acid) 15 mM, Tes (2-{[tris-(hydroxymethyl)-methyl]-amino} ethanesulphonic acid) 15 mM (Calbiochem, San Diego, CA, U.S.A.), and 10% newborn calf serum (Gibco), penicillin 35 μg ml^{-1} and streptomycin 50 μg ml^{-1} (Sigma) adjusted to pH 7.5. Release experiments were carried out 3 days after cell plating. Cells were washed twice in DMEM and finally 1 ml DMEM (control) or 1 ml DMEM with fenoldopam (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M) or quinpirole (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M), were added for 2 h, each in the presence of saline, domperidone (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M) or SCH 23390 (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M).

As SCH 23390 has been reported to have some affinity for 5-HT_2 receptors (Peroutka, 1988), we also investigated the possible involvement of 5-hydroxytryptamine receptors in prolactin secretion. Therefore, the 5-HT receptor antagonists, mianserin (10^{-6} M), ketanserin (10^{-6} M) or RU 24969 (10^{-6} M) were added for 2 h, together with fenoldopam (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M) in 1 ml DMEM.

At the end of incubation period, medium was removed and centrifuged at 2000 *g* in order to exclude cellular material. The supernatants were kept frozen at −20°C until prolactin hormone assay.

Membrane preparation and radioligand binding

Pituitary glands from 100 rats were dissected and kept on ice. All subsequent steps were performed at 4°C. Glands were homogenized in Tris-HCl 10 mM (pH 7.4), NaCl 145 mM, $MgCl_2$ 2 mM and sucrose 50 mM (Buffer A) with an Ultraturrax homogenizer (15 s at maximum speed) and a Potter Elvehjem homogenizer (5 up and down strokes at maximum speed). The homogenates were then centrifuged in a Sorvall centrifuge at 900 *g* for 5 min. The pellet was discarded and the supernatant was centrifuged subsequently 29,000 *g* for 20 min. The pellet was resuspended in Tris-HCl 10 mM (pH 7.4), NaCl 145 mM, $MgCl_2$ 2 mM (Buffer B) and centrifuged at 29,000 *g* for 20 min. This washing step was repeated twice. The final pellet was resuspended in buffer B and immediately used for binding assays. Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin (BSA) as a standard. Binding assays were done in duplicate in 0.5 ml of buffer B, containing 0.5 to 6 nM [^{3}H]-SCH 23390 or 0.03 to 2 nM [^{3}H]-spiperone and membrane protein (1.4 and 0.9 mg ml^{-1}, respectively). Mianserin (300 nM) was included to prevent binding of [^{3}H]-spiperone to 5-HT_2 receptors. After incubation of the membranes for 20 min at 30°C, 4 ml of ice cold buffer was added to each tube and samples were rapidly filtered through Whatman GF/B glass fibre filters (2.5 cm diameter). The filters were washed rapidly 3 times with 4 ml of ice cold buffer, placed in 20 ml polyethylene scintillation vials with 1 ml of 0.1 N NaOH and 10 ml of scintillation fluid (Pico Fluor 15 from Packard), and counted in a Packard liquid scintillation spectrometer.

Non-specific binding was determined in the presence of 10 μM (+)-butaclamol (for [^{3}H]-SCH 23390) or of 1 μM (+)-butaclamol (for [^{3}H]-spiperone). Specific binding was calculated by subtracting non-specific binding from total binding.

Autoradiographic studies

Radioligand bindings Dispersed rat pituitary cells were cultured for 3 days on 12 coverslips (15 mm Thermanox, Miles Laboratories Inc., Naperville, U.S.A.) as described previously (Velkeniers *et al.*, 1988). Each experiment was repeated twice. After washing in sodium phosphate 10 mM (pH 7.4)/NaCl 120 mM (Buffer C), 6 coverslips were slightly fixed with 0.5% glutaraldehyde and 0.04% paraformaldehyde in Buffer C for 15 min at 4°C. All 12 coverslips were then pre-incubated with sodium phosphate 20 mM (pH 7.4), NaCl 120 mM, $MgCl_2$ 2 mM (Buffer D) for 5 min at 4°C. Binding, as described by De Keyser *et al.* (1988a), was performed during 30 min at 30°C for fixed cells and during 60 min for 4°C for unfixed (i.e. living) cells in Buffer D, supplemented with 0.1% BSA (fraction V, Sigma, St. Louis, U.S.A.) and 100 KIU ml^{-1} aprotinin (Boehringer, Mannheim, West Germany). Radioligand concentrations were 0.5 nM for [^{3}H]-spiperone (83 Ci mmol^{-1}; Amersham), to detect D_2-receptors, and 2 nM for [^{3}H]-SCH 23390 (85 Ci mmol^{-1}; New England Nuclear) to detect D_1-receptors. Mianserin was included to mask binding to 5-HT_2 receptors and α_1-adrenoceptors: 300 nM for [^{3}H]-spiperone and 20 nM for [^{3}H]-SCH 23390. Non-specific binding was determined with unlabelled (+)-butaclamol (1 μM for

[^{3}H]-spiperone and 10 μM for [^{3}H]-SCH 23390), in addition to the respective radioligand. Specific binding was qualitatively evaluated with increasing fenoldopam concentrations, from 10^{-9} M to 10^{-6} M. After incubation, cells were washed twice with Buffer D for 5 min at 4°C to remove unbound ligand. Then ligand was fixed with 0.5% glutaraldehyde in Buffer C for 10 min at 4°C, as suggested by Junger (1978). After a brief rinse in cold distilled water, the coverslips were quickly air-dried and processed for immunocytochemistry.

Immunocytochemistry The avidin-biotin complex method (Hsu *et al.*, 1981) was used, employing a commercially available staining kit (Boehringer, Mannheim, West Germany). Buffer C (pH 7.2) was used as a diluent for all reagents and for the washes. The incubations with normal goat serum (10:100; 15 min) and with biotinylated goat anti-rabbit-IgG (1:100; 30 min) were performed at room temperature, except for rabbit-anti-prolactin (NIH; Bethesda, U.S.A.) (1:5000; 12 h overnight; 4°C). After incubation with the avidin-biotin complex, peroxidase activity was visualised with 3.3 diaminobenzidinetetrahydrochloride (0.15% w/v) and hydrogen peroxide (1% v/v) in Buffer C. Each incubation period, except the normal serum step, was followed by three washes of 5 min each.

Autoradiography For localization of [^{3}H]-SCH 23390 and [^{3}H]-spiperone, coverslips were dipped in liquid emulsion (nuclear K2 emulsion, Ilford) diluted with distilled water (1:1). After an exposure time of 4 weeks at −4°C, the autoradiograms were developed in Kodak D19 at 20°C for 8 min, rinsed in distilled water, fixed in Iford Rapid Fixer for 10 min and thoroughly washed in distilled water. Cells were then counterstained with haematoxylin to visualise cell nuclei. After mounting, coverslips were scanned with Axiophot (Zeiss, West Germany).

Adenylate cyclase assay

Harvested cells were centrifuged (1000 *g*) for 10 min at 4°C in Krebs-Ringer-HEPES buffer (composition mM: NaCl 110, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 25, HEPES 10 and BSA 1 mg ml^{-1}, pH 7.4). The pelleted cells were resuspended in the same buffer (10^6 cells ml^{-1}, final concentration). When indicated, cells were treated with drugs under culture conditions, centrifuged and resuspended in buffer as above. For cyclic AMP determinations, cells were incubated with 0.5 mM theophylline and the indicated compounds for 15 min at 37°C in a final volume of 200 μl. The incubation was stopped by addition of 100 μl of 4 mM EDTA (pH 7.4) and boiling for 6 min. Denaturated proteins were precipitated by centrifugation at 13,000 *g* for 2 min, and the cyclic AMP contents of the supernatants determined by the method of Gilman (1970), with protein kinase A used as binding protein.

Hormone assay and data analysis

Prolactin was measured by double antibody radioimmunoassay procedures, with use of anti-rat PRL antibodies and anti-rabbit-IgG, kindly provided by Dr Raiti (National Institute of Arthritis, Diabetes, Digestive Disease and Kidney). Rat prolactin was used as a standard.

The data were analysed by two-way analysis of variance with repeated measures. When significant F values occurred due to the treatment, the data were reanalysed by one-way analysis of variance, followed by the Student-Newman-Keuls procedure. A P value of less than 0.05 was considered significant. Data are expressed as means ± s.e.mean. Dose-response curves and 50% effective doses (ED_{50}) were calculated by use of weighted iterative nonlinear least squares regression.

Materials

The sources of the drugs used were as follows: (+)-butaclamol hydrochloride (Research Biochemicals Inc., Wayland, U.S.A.), mianserin (Organon, Oss, The Netherlands), fenoldopam (Smith Kline and French Laboratories, Philadelphia, U.S.A.), quinpirole hydrochloride (Lilly Research Laboratories, Indianapolis, U.S.A.), SCH 23390 [(R)-(+)-8chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine] (Schering Corporation, Bloomfield, U.S.A.) and RU 24969 (5-methoxy 3(1,2,3,6-tetrahydro-4-pyridinyl)1H indole succinate; Roussel-UCLAF, Paris, France). Spiroperidol, domperidone and ketanserin were kindly donated by Janssen Pharmaceutica (Beerse, Belgium). [^{3}H]-SCH 23390 (80 Ci mmol^{-1}) was supplied by Amersham (U.K.) and [^{3}H]-spiperone (24 Ci mmol^{-1}) by New England Nuclear. All other chemicals were of the highest grade commercially available.

Results

In vivo *experiments*

Saline, fenoldopam or quinpirole did not significantly change basal serum prolactin levels in conscious rats (4.21 ± 2.63 ng ml^{-1} before and 4.39 ± 2.11 ng ml^{-1} 40 min after injection of saline, $n = 6$; 4.85 ± 2.35 before and 4.17 ± 1.99 ng ml^{-1} after fenoldopam, $n = 6$; and 3.84 ± 1.72 ng ml^{-1} before and 3.02 ± 2.12 ng ml^{-1} after quinpirole, $n = 6$).

AMPT increased serum prolactin from 4.34 ± 2.88 to 48.6 ± 25.6 ng ml^{-1} ($n = 96$) after 1 h and it remained elevated for at least 6 h. In AMPT-treated rats, saline or SCH 23390 did not significantly change serum prolactin. Quinpirole significantly and dose-dependently decreased prolactin secretion ($ED_{50} = 4\ \mu g\ kg^{-1}$) (Figure 1). Fenoldopam also significantly reduced prolactin secretion in a dose-dependent manner ($ED_{50} = 24\ \mu g\ kg^{-1}$) (Figure 1). The maximal inhibitory effect of fenoldopam was significantly less pronounced than that of quinpirole (59.8 ± 3.5% vs. 93.0 ± 2.3%, $P \leqslant 0.01$). Pretreatment with SCH 23390 ($100\ \mu g\ kg^{-1}$) reduced the effect of fenoldopam (prolactin levels were 40.7 ± 15.2 before and 35.6 ± 19.6 ng ml^{-1} after administration of fenoldopam with SCH 23390, $n = 6$), but did not affect basal nor quinpirole-inhibited prolactin secretion (Figure 1).

In vitro *experiments*

Both, fenoldopam and quinpirole dose-dependently reduced prolactin secretion from cultured pituitary cells (Figure 2a,b). SCH 23390 provoked a dose-dependently rightward shift of the dose-response curve of fenoldopam (Figure 2a), but did

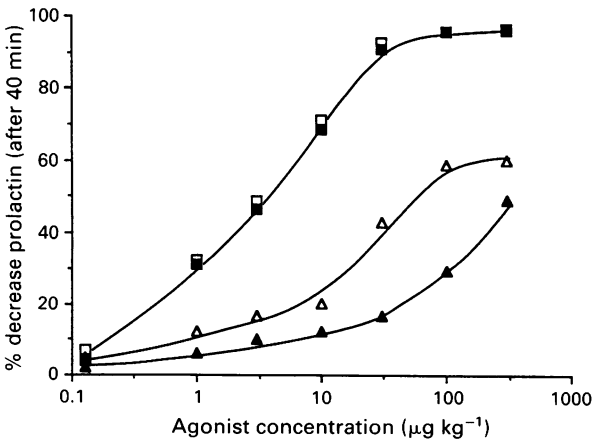

Figure 1 Concentration-response relationships for the effects of quinpirole (□), fenoldopam (△), quinpirole plus SCH 23390 (■) and fenoldopam plus SCH 23390 (▲) on prolactin secretion *in vivo* after pretreatment with α-methyl-*p*-tyrosine. Responses are calculated as a % decrease from the baseline level. Values are expressed as means from 6 to 12 experiments. Standard errors of the mean are not shown for reasons of clarity, but were less than 4.1%.

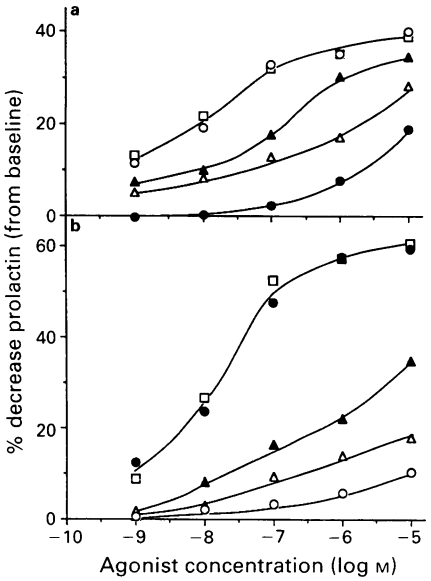

Figure 2 Prolactin secretion from cultured pituitary cells: effect of increasing concentrations of fenoldopam (a) and quinpirole (b). In the experiments with fenoldopam, cells were pretreated with saline (□), domperidone (10^{-6} M, ○), SCH 23390 (10^{-6} M, ●; 10^{-7} M, △ or 10^{-8} M, ▲). In the experiments with quinpirole, cells were pretreated with saline (□), SCH 23390 (10^{-6} M, ●), domperidone (10^{-6} M, ○; 10^{-7} M, △ or 10^{-8} M, ▲). Responses are calculated as a % decrease from the baseline value. Values are expressed as mean; $n = 12$. Standard errors of the mean are not shown for reasons of clarity, but were less than 2.8%.

not affect the dose-response curve of quinpirole (Figure 2b). Domperidone did not antagonize the prolactin lowering effect of fenoldopam (Figure 2a), but dose-dependently inhibited the effect of quinpirole (Figure 2b). Schild plot analysis revealed a pA_2 value of 8.83 for SCH 23390 (slope of the Schild regression = 0.96 and linearity, represented by $r = 0.98$) and a pA_2 value of 9.39 for domperidone (slope = 0.98 and $r = 0.97$).

Agents acting on 5-HT receptors have been found to affect prolactin secretion *in vivo* (Seeman & Grigoriadis, 1987). Therefore, we examined the ability of three 5-HT antagonists, mianserin, ketanserin and RU 24969 (each in a concentration of 10^{-6} M), to inhibit the prolactin lowering effect of fenoldopam. None of the antagonists had a significant effect on basal prolactin secretion or fenoldopam-induced inhibition of prolactin secretion.

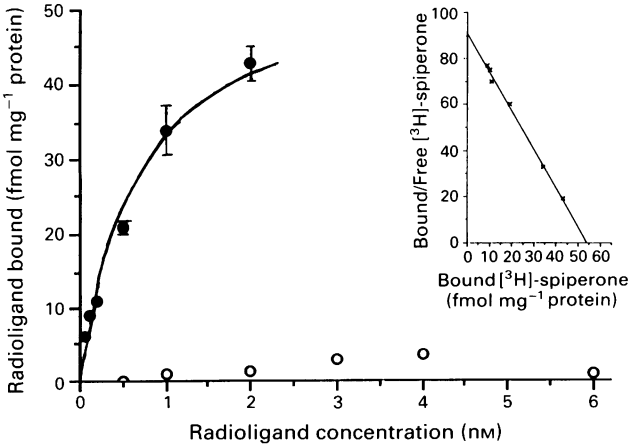

Figure 3 Specific binding of [^{3}H]-SCH 23390 and [^{3}H]-spiperone to pituitary membranes. Crude membrane preparations were incubated with increasing concentrations of radioligand as described in Methods. Data shown (means ± s.d. of 2 determinations) refer to specific binding of [^{3}H]-SCH 23390 (○) and [^{3}H]-spiperone (●). Inset: Scatchard transformation of [^{3}H]-spiperone binding to pituitary membranes. Transformation of binding yielded a linear plot: K_D 0.58 nM; B_{max} 54 fmol mg^{-1} protein.

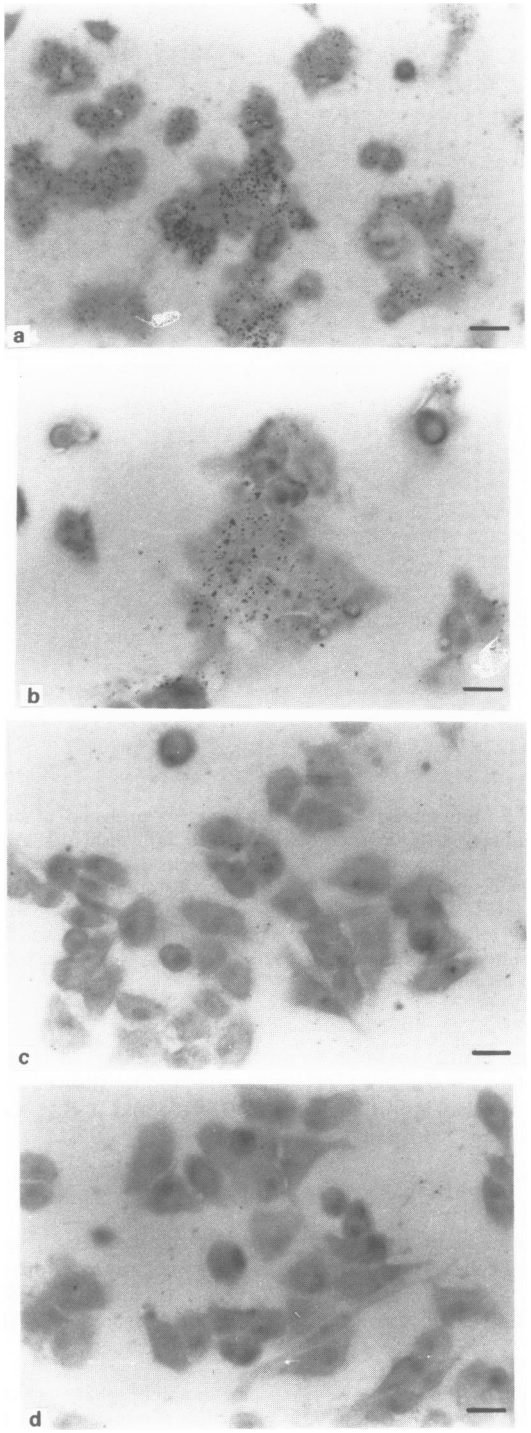

Figure 4 Autoradiographic localization of [^{3}H]-SCH 23390 and [^{3}H]-spiperone. After 4 weeks of exposure, binding sites are visualized by black dots. Cells were counterstained with haematoxylin to visualise cell nuclei. Specific binding revealed D_2-receptors (a) and D_1-receptors (b). Non-specific binding was negative in both cases: (c) for D_2-receptors, and (d) for D_1-receptors. Scale bar represents 20 μm.

Radioligand binding

No specific binding of [^{3}H]-SCH 23390 (in a dose from 0.5 to 6 nM) was detected in crude membrane preparations of pituitary glands (Figure 3). Under the same experimental conditions, high affinity specific binding of [^{3}H]-spiperone (from

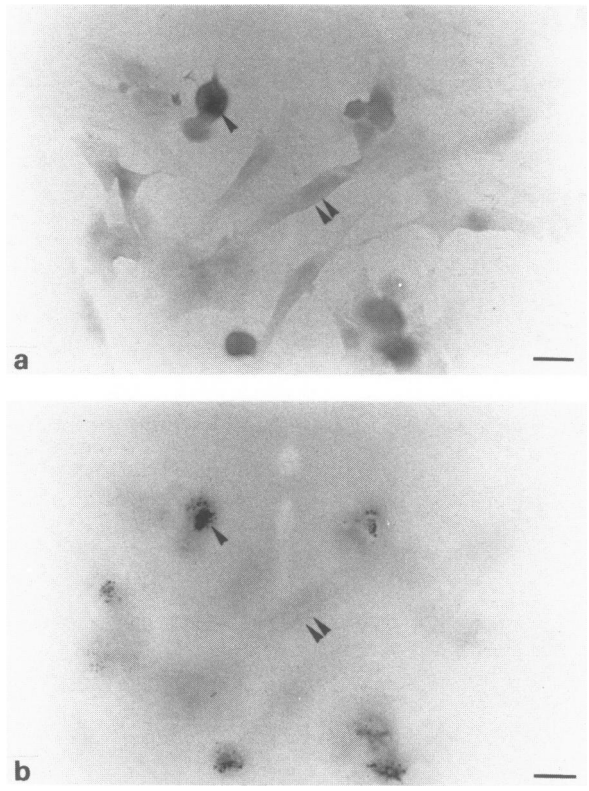

Figure 5 Immunocytochemical labelling for prolactin of dispersed rat pituitary cells. Prolactin cells stained brownish (one-arrow, a), whereas immunocytochemical negative cells (two arrows, a) were identifiable only by their blue nucleus. Autoradiographic labelling (with [^{3}H]-SCH 23390) was only present on immunocytochemical positive cells (one arrow, b), whereas immunocytochemical negative cells were unlabelled with silver grains (two arrows, b). Scale bar represents 20 μm.

0.05 to 2.0 nM) to these membranes was observed (Figure 3). Scatchard analysis of [^{3}H]-spiperone binding showed an equilibrium dissociation constant of 0.58 nM and a B_{max} value of 54 fmol mg^{-1} protein.

Autoradiographic studies

No differences in labelling were found between fixed and unfixed cells. Non-specific binding was negative in both conditions. Specific binding sites for both [^{3}H]-SCH 23390 and [^{3}H]-spiperone were demonstrated on cultured cells (Figure 4). More cells were labelled for D_2-receptors than for D_1-receptors. Fenoldopam, in doses of 1 nM up to 1 μM, dose-dependently reduced the number of positive cells for [^{3}H]-SCH 23390, but had no effect on [^{3}H]-spiperone binding. Immunocytochemical staining of prolactin cells, after incubation with [^{3}H]-SCH 23390 or [^{3}H]-spiperone, is shown in Figure 5. Pituitary cells, staining for prolactin, also contained the granulae, representing [^{3}H]-SCH 23390 binding.

Table 1 Adenosine 3':5'-cyclic monophosphate (cyclic AMP) contents of pituitary cells: effect of prostaglandin E_1 (PGE_1), dopamine and fenoldopam

Addition	*Cyclic AMP* (pmol/10^6 cells)
None	2.1 ± 0.3
PGE_1	6.2 ± 0.5
Dopamine (0.1 μM)	4.8 ± 0.4
Dopamine (1 μM)	4.4 ± 0.3
Dopamine (10 μM)	4.4 ± 0.2
Dopamine (100 μM)	4.1 ± 0.2
Dopamine (1 mM)	4.0 ± 0.2
Fenoldopam (0.1 μM)	2.1 ± 0.2
Fenoldopam (1 μM)	2.3 ± 0.3
Fenoldopam (10 μM)	2.0 ± 0.2
Fenoldopam (100 μM)	2.4 ± 0.2
Fenoldopam (1 mM)	2.3 ± 0.2

Cells were treated for 15 min at 37°C with the indicated compounds after which the cyclic AMP content was measured as described in Methods. Values are means ± s.e.mean of 4 determinations.

Effect on basal adenylate cyclase

Basal cyclic AMP production of the dispersed rat pituitary cells was 2.1 pmol/10^6 cells, and increased to 6.2 pmol/10^6 cells after administration of prostacyclin (PGE_1, 20 μM) for 15 min. Under these experimental conditions, dopamine (0.1 μM, 1 μM, 10 μM, 100 μM and 1 mM) dose-dependently inhibited cyclic AMP production. Fenoldopam (0.1 μM, 1 μM, 10 μM, 100 μM and 1 mM) did not affect cyclic AMP formation (Table 1).

Discussion

Our results confirm and extend previous work showing the presence of specific D_2-receptors on lactotroph cells, involved in prolactin secretion (Foord *et al.*, 1983; Enjalbert *et al.*, 1986). We and others (Dupont *et al.*, 1986; Boesgaard *et al.*, 1989) were unable to demonstrate any effect of fenoldopam, the most selective D_1-receptor agonist currently available, on prolactin secretion in *in vivo* base-line conditions. This is in agreement with the currently accepted theory that the anterior pituitary dopamine receptors are of the D_2-type (Caron *et al.*, 1978; Sibley *et al.*, 1982; Dumbrille-Ross & Seeman, 1987) and that prolactin secretion is tonically inhibited by dopamine and that D_2-receptor agonists, such as bromocriptine (Delpozo *et al.*, 1972) and quinpirole (Lefevre-Borg *et al.*, 1987) can lower prolactin. Blockade of the hypothalamic dopaminergic influence, by AMPT, resulted in an immediate increase in plasma prolactin. When the *in vivo* experiments were repeated after pretreatment with AMPT, fenoldopam, as well as quinpirole, provoked a significant and dose-dependent reduction of prolactin secretion. SCH 23390, a selective D_1-receptor antagonist (Hyttel, 1983), abolished the effect of fenoldopam, suggesting that D_1-receptors are also able to modulate the secretion of prolactin. The prolactin lowering effect of fenoldopam was less pronounced than that of quinpirole. This difference might be related to several factors, such as the number of D_1- and D_2-receptors, the intrinsic activity of the agonists used and the differences in the efficiency of the receptor-effector coupling mechanisms. In subsequently performed *in vitro* experiments we were able to demonstrate that both fenoldopam and quinpirole dose-dependently reduced prolactin secretion from cultured pituitary cells. These data indicate that this D_1- receptor is localized on a pituitary cell rather than an extra-pituitary site (i.e. hypothalamic or at the portal vascular bed), as suggested by Cocchi *et al.* (1987). The pA_2 values for SCH 23390 and for domperidone, obtained by Schild plot analysis, are in agreement with the affinity of these drugs for D_1- and D_2-receptors respectively (Seeman & Grigoriadis, 1987). The 5-HT receptor antagonists used in this study did not significantly affect the fenoldopam-induced inhibition of prolactin secretion. Thus, the 5-HT_{1A}, 5-HT_{1B} and 5-HT_2 receptor subtypes (Peroutka, 1988) are not involved. This is in agreement with the D_1-selectivity of fenoldopam (Hahn *et al.*, 1982) and the fact that no 5-HT_2 receptors have been detected in the pituitary so far.

The dopamine receptors in anterior pituitary membranes have been studied in the past with different radiolabelled ligands, such as dihydroergocryptine, spiperone and dopamine itself (Creese *et al.*, 1983). In all these studies this binding site fulfilled the criteria of a D_2-receptor. In this study, we also detected high affinity binding sites for [^{3}H]-spiperone in a crude membrane preparation from pituitary glands. The

binding parameters (K_d and B_{max}) are similar to those previously reported for pituitary D_2-receptors (Seeman & Grigoriadis, 1987). In agreement with the study of Rovescalli *et al.* (1987) no specific binding for [^{3}H]-SCH 23390 could be demonstrated, indicating that no binding sites for [^{3}H]-SCH 23390 are present or that the binding site is lost during preparation. This latter explanation is favoured by the autoradiographic studies on cultured cells, which revealed specific binding sites for both [^{3}H]-spiperone and [^{3}H]-SCH 23390. As fenoldopam dose-dependently reduces [^{3}H]-SCH 23390 binding and has no effect on [^{3}H]-spiperone binding, the binding sites for [^{3}H]-SCH 23390 appear to correspond to D_1-receptors, rather than to D_2-receptors. [^{3}H]-SCH 23390, as well as [^{3}H]-spiperone, granulae were present on lactotroph cells, as demonstrated by immunochemical labelling, suggesting that D_1- and D_2-binding sites are located on the prolactin secreting cells.

In view of the initial assumption that D_1- and D_2-receptors exert an opposite control on the adenylate cyclase activity (Swennen & Denef, 1982; Stoof & Kebabian, 1981), it seems contradictory that both receptors are capable of inhibiting prolactin release. However, D_1- and D_2-receptors have been described as controlling other membrane signalling pathways as well (Andersen *et al.*, 1990); indeed, the chain of molecular events, linking receptor stimulation to inhibition of prolactin release, still remains to be elucidated. D_1-receptors have for example been shown to stimulate the phosphadityl inositol turnover pathway (Berridge, 1987); the proximal tubular D_1-receptor e.g. has been shown to stimulate phospholipase-C activity independent of adenylate cyclase (Felder *et al.*, 1989). Moreover, a D_1-receptor, not linked to adenylate cyclase, has recently also been identified in the amygdala (Mailman *et al.*, 1986), the stellate ganglia (Sabouni *et al.*, 1987) and the human frontal cortex (De Keyser *et al.*, 1988b). In this context, the failure of fenoldopam to increase the cyclic AMP levels in pituitary cells might reflect linking of pituitary D_1-receptors to an alternative membrane signalling pathway. Dopamine itself did not inhibit cyclic AMP formation of dispersed rat pituitary cells, which can be explained by the relatively low basal level of cyclic AMP. Malfunctioning of the adenylate cyclase system was ruled out on basis of the ability of PGE_1 to produce a 3 fold increase in the cellular cyclic AMP levels.

We conclude from our pharmacological, radioligand binding, autoradiographic and adenylate cyclase studies that, in contrast to the report of Kebabian & Calne (1979), the anterior pituitary gland contains more than one type of dopamine receptor. It is tempting to speculate that fenoldopam reduces prolactin secretion through interaction with a non-cyclase-linked D_1-receptor on the lactotroph cells.

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Blockade of intracellular actions of calcium may protect against ischaemic damage to the gerbil brain

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1 The brain cytoprotective effects of a putative calcium-associated protein kinase inhibitor, HA1077, as well as a calcium entry blocker nicardipine were evaluated in models of cerebral ischaemia in Mongolian gerbils. Morphological changes characterizing delayed neuronal death of selectively vulnerable CA_1 pyramidal neurones in the hippocampus of the Mongolian gerbil brain occurred 7 days after transient bilateral occlusion of the common carotid arteries.

2 A single injection of HA1077 (1 and 3 mg kg^{-1}, i.p.) 5 min after the occlusion led to a dose-dependent protection of the CA_1 neurones. Repeated administrations of HA1077 (1 and 3 mg kg^{-1}, i.p., twice daily for 7 days post-ischaemia) revealed an increase in the number of normal cells, compared to findings with a single administration.

3 In contrast to HA1077, nicardipine (0.3 and 1 mg kg^{-1}, i.p.) did not reduce neuronal degeneration.

4 HA1077 did not interact with the ion channel within which MK-801 binds, as determined by receptor binding.

5 The calcium ionophore, A23187, caused a tonic contraction in canine cerebral arterial strips. HA1077, but not nicardipine, relaxed the A23187-induced contraction, concentration-dependently.

6 These results suggest that blockade of the intracellular actions of calcium may provide protection against ischaemic damage in the brain.

Keywords: Delayed neuronal death; ischaemia; calcium antagonists; protein kinase; HA1077

Introduction

Kirino (1982) described a gerbil model of forebrain ischaemia, induced by transient occlusion of both common carotid arteries, the result being a reproducible discrete lesion featuring extensive neuronal degeneration in the CA_1 subfield of the hippocampus, in 90% of the experiments. It takes several days for this neuronal damage to become apparent at the light microscopic level, hence this phenomenon was termed 'delayed neuronal death' (Kirino, 1982). A similar temporal profile of neuronal ischaemic damage in rats subjected to transient forebrain ischaemia has been described by Pulsinelli *et al.* (1982). It has been suggested that delayed neuronal death is mediated by the following events: (1) excessive release of glutamate from nerve terminals during the ischaemic period; (2) activation of N-methyl-D-aspartate (NMDA)-type glutamate receptors, and (3) influx of Ca^{2+} into the neurones, probably through NMDA receptor-linked calcium channels. Although factors following these events are not clear, nor is the precise mechanism of the delayed onset of the neuronal damage well understood, both require attention when attempting to design treatment for postischaemic brain damage in man.

The pathological accumulation of intracellular calcium is thought to be one factor that causes or accompanies cell death (Siesjo, 1981). Neuronal cell damage observed after transient cerebral ischaemia was found to correlate strongly with an intracellular accumulation of calcium (DeLeo *et al.*, 1987).

Attention has been given to various calcium modulators for their potential experimental and clinical usefulness in the therapy of disorders related to cerebral ischaemia.

Thus, we examined a novel calcium antagonistic vasodilator HA1077 (1-5-(isoquinolinesulphonyl)-homopiperazine HCl), proposed as a calcium-associated protein kinase inhibitor (Asano *et al.*, 1989), and nicardipine, a calcium entry blocker which prevents ischaemia-induced cell death in hippocampal neurones of Mongolian gerbils.

HA1077 was found to produce a competitive inhibition of Ca^{2+}-induced contractions of strips of depolarized rabbit aorta and significantly inhibited phenylephrine-induced contraction in Ca^{2+}-free solution (Asano *et al.*, 1987). The contraction in response to adrenoceptor stimulation in Ca^{2+}-free solution seems to be due almost entirely to the release of intracellular Ca^{2+} (Fleckenstein, 1977). HA1077 did not alter the developed tension in the guinea-pig left atrium, hence, unlike calcium entry blockers, HA1077 does not have a blocking action on slow myocardial calcium channels (Asano *et al.*, 1987). Cultured vascular smooth muscle cells exposed to HA1077 showed a dose-dependent loss or a decrease in size and length of the actin-containing microfilament structure (Sasaki *et al.*, 1987). Neither verapamil nor diltiazem induced reorganization of the actin microfilament structure. HA1077 potently inhibited calcium-associated protein kinases considered to have an important role in regulation of cellular function (Asano *et al.*, 1989). These results suggest that the mechanism of action of HA1077 differs from that of conventional calcium entry blockers and that its effect may depend on actions on internal sequestering sites.

Methods

Delayed neuronal death study

Male Mongolian gerbils, weighing 55 to 80 g, were placed in the supine position. Under light anaesthesia with ether, both common carotid arteries were exposed through a ventral midline incision and separated carefully from the adjacent vein and sympathetic nerves. The arteries were then clamped with aneurysm clips. After 5 min, the clips were removed and the skin was sutured. Sham-operated animals were treated in the same manner, except for no clamping.

Test drug solution or saline (0.9% w/v NaCl) was injected i.p. 5 min after artery occlusion in the single injection groups. After the single injection, in addition, repeat injection groups

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were given doses of the respective test drug twice daily at intervals of 12 h during the post-ischaemia period.

On day 7 after a 5 min occlusion, the animals used for the histopathological examination were anaesthetized with pentobarbitone and the brains were perfused with a 10% buffered formalin solution given through the left cardiac ventricle. The brains were dissected out and fixed in 10% buffered formalin solution. The hippocampal region was cut coronally into 3 to 4 mm thick slices, embedded in paraffin and processed with the step section technique. The slides were stained with haematoxylin and eosin and cresyl violet.

The pyramidal cells of each CA_1 region were counted under a light microscope at $\times 200$ magnification. The mean number of CA_1 pyramidal neurones per millimetre for both hemispheres, (the neuronal density), was calculated for each group, according to Izumiyama & Kogure (1988).

Vascular relaxation study

Mongrel dogs were anaesthetized with sodium pentobarbitone and exsanguinated from the femoral artery. Basilar arteries were rapidly removed and kept on crushed ice in oxygenated Krebs-Henseleit solution (mM: NaCl 115, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 2.5, KH_2PO_4 1.2 and dextrose 10.0). Each artery was helically cut into equal strips 20–25 mm long and 1–2 mm wide and the strips were suspended under a resting tension of 1.0 g in a 20 ml tissue bath containing Krebs-Henseleit solution at 37°C; they were aerated continuously with a mixture of 95% O_2 and 5% CO_2. Changes in arterial tension were recorded isometrically through a force-displacement transducer (Nihon Kohden, SB-1T) and a polygraph.

After 60–90 min of equilibration, 40 mM KCl was added to the bath two or three times until successive responses of the tissue remained constant. Helical strips of the basilar artery were contracted with 3×10^{-6} M A23187, and when the contraction had reached a plateau, HA1077 or nicardipine was added.

NMDA receptor antagonism study

After male Sprague-Dawley rats (200–250 g, Charles River) were killed by cervical dislocation their brains were removed and rapidly dissected at 4°C to obtain cerebral cortex and hippocampus. All further procedures were carried out at 4°C. The cerebral cortex and hippocampus were homogenized separately in 30 volumes of 10 mM HEPES buffer (pH 7.4) with a Teflon-glass homogenizer. The homogenate was centrifuged at 48,000 *g* for 15 min. The pellet was resuspended in 30 volumes of distilled water and lysed at 37°C for 20 min followed by centrifugation at 48,000 *g* for 15 min. This process was repeated once more. The resulting pellet was resuspended in 30 volumes of 10 mM HEPES containing 1 mM EDTA and frozen at −80°C until use in binding experiments.

On the day the binding assay was performed, tissue suspensions were thawed, homogenized in 100 volumes (with respect to original tissue weight) of 10 mM HEPES and centrifuged at 48,000 *g* for 15 min. The pellet was resuspended in 10 volumes of 10 mM HEPES buffer. All preparative procedures and binding assays were performed in ultrapure water.

For [^{3}H]-MK-801 binding assays, the tissue preparations were incubated in duplicate with 5 nM [^{3}H]-MK-801 in the presence of various concentrations of unlabelled drugs, 10^{-5} M glutamate and glycine. Incubations were carried out for 60 min at 25°C in a final volume of 1 ml. Nonspecific binding was determined with 10^{-5} M phencyclidine. The binding reaction was terminated by filtration under vacuum through Whatman GF/C glass fibre filters. Filters were washed twice with 5 ml of 10 mM HEPES buffer and the retained radioactivity determined using a liquid scintillation counter.

Statistics

The values are given as mean ± s.e.mean. The significance of difference was calculated by Student's *t* test or Duncann's multiple range test. *P* values of 0.05 or less were considered to represent significant differences.

Drugs

The drugs used were nicardipine HCl (Yamanouchi, Tokyo, Japan), [^{3}H]-MK-801 (New England Nuclear, Boston, MA, U.S.A.) and phencyclidine (Sigma, St Louis, MO, U.S.A.).

Results

Delayed neuronal death study

Complete forebrain ischaemia for a period of 5 min resulted in a model that showed consistent neuronal degeneration, in

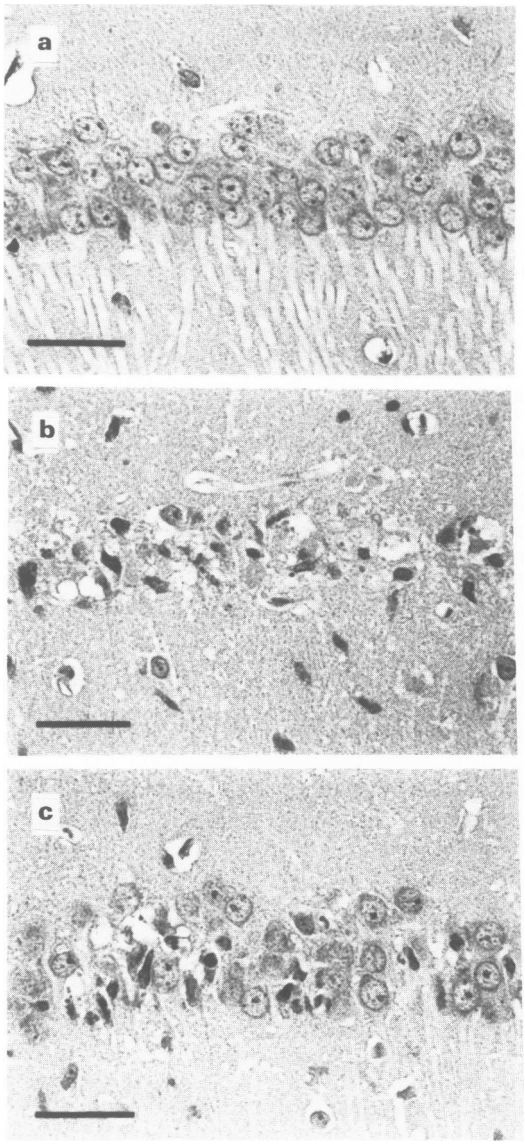

Figure 1 Light micrographs of coronal sections stained with haematoxylin and eosin and showing morphological changes on day 7 following transient ischaemic insult. Bar represents 20 μm. (a) Pattern of degeneration of pyramidal cells in CA_1 area in the hippocampus of a sham-operated gerbil. (b) Shows significant loss of structural integrity and neuronal death in a saline-treated animal subjected to 5 min bilateral carotid occlusion. Ghost cell spaces, darkly stained shrunken dead cells and cellular debris are characteristic features of delayed neuronal death. (c) Significant protection of pyramidal cells in the hippocampus of a gerbil given repeated injections of HA1077 (3 $mg \cdot kg^{-1}$, i.p., twice daily) during ischaemia.

Table 1 Effects of drug treatment on delayed neuronal death in the hippocampus CA_1 subfield of the gerbil brain

Treatment	*Normal cell count* (cells mm^{-1}) *Single injection*	*Repeat injection*
Saline	17.8 ± 2.1 ($n = 14$)	17.4 ± 2.8 ($n = 10$)
HA1077 1 mg kg^{-1}	34.4 ± 6.6* ($n = 15$)	71.3 ± 23.9* ($n = 11$)
HA1077 3 mg kg^{-1}	40.0 ± 9.9* ($n = 15$)	92.0 ± 17.3**,† ($n = 10$)
Nicardipine 0.3 mg kg^{-1}	29.5 ± 5.7 ($n = 10$)	
Nicardipine 1 mg kg^{-1}	28.0 ± 6.4 ($n = 10$)	

Saline or test drug solution was injected i.p.
Values are mean ± s.e.mean (n = numbers of animals).
* $P < 0.05$; ** $P < 0.01$ vs. each saline treatment group.
† $P < 0.05$ vs. each single injection group.

each animal. The pattern of neurodegeneration involved virtually all pyramidal neurones in the CA_1 areas of both hippocampus on day 7 (Figure 1). Assessment of the hippocampal damage by measurement of the normal pyramidal neurones of the CA_1 region proved to be a convenient and highly consistent approach. In the sham-operated gerbils, the normal neurone count was 213 ± 5 cells mm^{-1} ($n = 11$). In the saline-treated animal, the normal cell count was significantly different from sham-operated animals on day 7.

Table 2 Relaxation of A23187-induced contraction by HA1077 in canine basilar arteries

	Tension (mg)	n
HA1077 treatment		
A23187alone	1605 ± 89	8
+ HA1077 10^{-6} M	1326 ± 93**	8
+ HA1077 10^{-5} M	816 ± 89**	8
+ HA1077 10^{-4} M	681 ± 89**	8
Nicardipine treatment		
A23187 alone	1789 ± 259	3
+ nicardipine 10^{-6} M	1798 ± 251	3
+ nicardipine 10^{-5} M	1834 ± 230	3

HA1077 or nicardipine were added to helical strips of canine basilar artery precontracted with 3×10^{-6} M A23187. HA1077 or nicardipine were added after peak tension had been achieved. Values are mean ± s.e.mean. n = numbers of animals.
** $P < 0.01$ by Student's paired t test vs. control group.

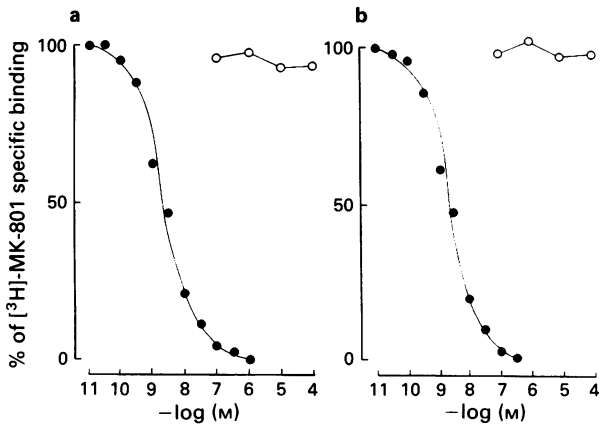

Figure 2 Effect of HA1077 (○) and MK-801 (●) on [^{3}H]-MK-801 binding in the rat cerebral cortex (a) and hippocampus (b). Each point represents mean value for 2 determinations performed in duplicate. Assays contained 10^{-5} M glutamate and glycine.

A single injection of HA1077 (1 and 3 mg kg^{-1}, i.p.) 5 min after bilateral occlusion led to a dose-dependent increase in the normal cell count in the CA_1 region (Table 1). In contrast, nicardipine (0.3 and 1 mg kg^{-1}, i.p.) did not reduce the area of neuronal degeneration in the hippocampus.

Repeat injection of HA1077 (1 and 3 mg kg^{-1}, i.p., twice daily) also significantly reduced nerve cell damage, in a dose dependent manner (Figure 1 and Table 1).

Morphological findings of the repeat saline-treated groups were similar to those of the single saline-treated groups. However, repeat injection of HA1077 (3 mg kg^{-1}, i.p., twice daily) significantly increased the number of normal cells compared to findings in the single injection group. Although there were large differences in the mean values in the HA1077 1 mg kg^{-1}-treated group, the differences were not statistically significant.

Vascular relaxation study

The canine basilar artery contracted with calcium ionophore A23187 responded differently to HA1077 and nicardipine, respectively (Table 2). Cumulative increase in the concentration of HA1077 (10^{-6} M to 10^{-5} M) added to canine basilar arterial strips contracted by 3×10^{-6} M A23187 resulted in a concentration-dependent relationship for relaxation. Conversely the same procedure in the concentration of nicardipine (10^{-6} M to 10^{-5} M) had no effect on the contraction produced by A23187.

NMDA receptor antagonism study

HA1077 did not inhibit the specific binding of the NMDA-type receptor antagonist [^{3}H]-MK-801 in the rat cerebral cortex or hippocampus preparations (Figure 2).

Discussion

The present results show that a novel calcium antagonist and vasodilator, HA1077, reduces the incidence of delayed neuronal death in the CA_1 subfield of the gerbil hippocampus following transient bilateral carotid artery occlusion.

The present NMDA receptor antagonism study relied on the high specificity of MK-801 so that a crude homogenate could be used. As shown in Figure 2. HA1077 clearly differed from a noncompetitive NMDA receptor channel blocker, MK-801. HA1077 had no effect on MK-801 binding. This is not indicative of a lack of effect at the NMDA receptor, but a lack of effect on the ion channel within which MK-801 binds, since binding was performed at equilibrium and in the presence of high concentrations of glutamate and glycine. Glutamate (10^{-5} M) was included, to activate fully the NMDA receptor and allow full access to the open channel. The glycine level was held high for the same reason. Furthermore, we have used cold MK-801 to displace ^{3}H-labelled MK-801. In a subsequent study, use of another ligand (e.g. TCP) may be preferable.

The accumulation of intracellular calcium is thought to be a major triggering event in the development of tissue damage following ischaemia in the brain (Siesjo, 1981). In attempts to prevent the harmful effects associated with Ca^{2+} influx, a block or decrease the influx of calcium by use of calcium entry blockers has been examined. Some investigators reported that these agents may decrease cerebral damage (Steen *et al.*, 1983; Alps *et al.*, 1988) while no beneficial effect has also been noted (Steen *et al.*, 1984).

Alps *et al.* (1988) administered calcium entry blockers such as nicardipine and nimodipine before 5 min-carotid artery occlusion in the Mongolian gerbil. The overall protection afforded by nicardipine (0.5 mg kg^{-1}, i.p.) was statistically significant. Nimodipine (0.5 mg kg^{-1}, i.p.) was inactive in this

model (Alps et al., 1988). This beneficial effect of pre-ischaemia treatment with nicardipine may be due to a block of the influx of Ca^{2+}. The question of treatment with nicardipine during the post-ischaemic period in gerbils has not been documented. In the present study, nicardipine (0.3 and 1 mg kg^{-1}, i.p.) administered after bilateral occlusion led to no decrease in neurodegeneration. This discrepancy may be related to the different protocols (pre-ischaemia administration vs. post-ischaemia administration). A distrubance in calcium homeostasis was evident in the gerbil hippocampus after a brief transient ischaemia (Sakamoto et al., 1986). We assumed that the intracellular calcium concentration in nerve cells had been raised, when nicardipine was administered after an ischaemic event. Thus, post-ischaemic treatment with nicardipine could not protect against neuronal degeneration.

Calcium ionophore, A23187, transports calcium as a lipid soluble complex through a number of natural and artificial membranes and increases intracellular calcium concentrations (Schaffer et al., 1973). In contrast to nicardipine, HA1077 relaxed the A23187-induced contraction of basilar artery strips. The present result is in agreement with the previous studies which suggested that HA1077, unlike calcium entry blockers, can block the intracellular actions of calcium (Asano et al., 1987; 1989; Sasaki et al., 1987).

Of greater interest is the decrease in brain damage when HA1077 was administered following ischaemia. We suggest that the brain cytoprotective effect of HA1077 is related to its ability to block the intracellular actions of calcium as discussed above, i.e., HA1077 could block adverse reactions detrimental to the cell triggered by excessive intracellular calcium after ischaemia.

In support of our working hypothesis, we found that HA1077 potently inhibits certain calcium related-protein kinases (Asano et al., 1989). For example, the K_i value of HA1077 for guanosine 3':5'-cyclic monophosphate (cyclic GMP) or adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase was 1.6×10^{-6} M. The concentrations of HA1077 inhibiting agonist-induced vascular contractions were similar to those inhibiting protein kinase. The potent kinase antagonistic effects of HA1077 are likely to be related to the blocking action of intracellular calcium during ischaemia and contribute to the anti-ischaemic effect of HA1077. It should be noted that ischaemia, hypoxia, and NMDA produce increases in cerebellar cyclic AMP or cyclic GMP production (Yoshida et al., 1980; Siesjo, 1981). The involvement of calcium-related protein kinases in the evolution and consequences of cerebral ischaemia remains to be determined.

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Inotropic and electrophysiological effects of BDF 9148, a congener of DPI 201-106, in guinea-pig atria and papillary muscles

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1 BDF 9148 is a newly synthesized congener of DPI 201-106 in which the piperazidinyl moiety has been replaced by an azetidine-3-oxy-moiety. The inotropic effect of both drugs was studied as well as their influence on the action potential, by use of standard microelectrode techniques.

2 BDF 9148 increased the contractile force of guinea-pig atria and papillary muscles. The EC_{50} was 1.32×10^{-7} mol l^{-1} and 0.7×10^{-6} mol l^{-1} respectively. DPI 201-106 was effective in a similar concentration-range, the EC_{50} being 2.6×10^{-7} mol l^{-1} for atria and 2.8×10^{-7} mol l^{-1} for papillary muscles.

3 Both drugs caused a concentration-dependent prolongation of the relaxation time of the isometric contraction curve. With 10^{-6} mol l^{-1} BDF 9148, the mean increase was 39.1 ± 4.4 ms in atria and 39.4 ± 7.5 ms in papillary muscles while 10^{-6} mol l^{-1} DPI 201-106 caused increases of 56.7 ± 2.5 ms and 79.3 ± 11.7 ms, respectively. The effect of BDF 9148 was not prevented by propranolol, but was reversed by 3×10^{-6} mol l^{-1} tetrodotoxin. Pretreatment of atria with 3×10^{-8} mol l^{-1} BDF shifted the concentration-response curve of ouabain to the left and reduced the EC_{50} of the glycoside from 3.21×10^{-7} mol l^{-1} to 2×10^{-7} mol l^{-1}.

4 BDF 9148 and DPI 201-106 concentration-dependently increased the action potential duration of papillary muscles and their functional refractory period. The drugs did not modify the resting potential, the action potential amplitude or the maximum depolarization velocity.

5 All effects of BDF 9148 persisted after washout. The lipophilic drug accumulated in the tissues and the tissue drug concentration was little reduced by washout of BDF 9148 from the organ bath.

6 In contrast to DPI 201-106, which had a prominent negative chronotropic effect in right atria, BDF 9148 caused only a slight reduction of the beating frequency with the largest (3×10^{-5} mol l^{-1}) concentration.

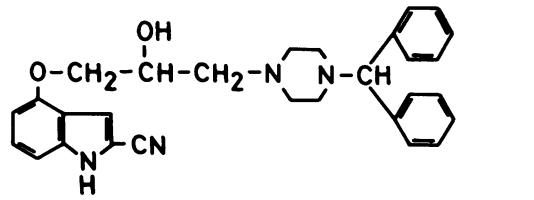
7 The results are consistent with BDF 9148 being a sodium channel activator with much weaker influence on the beating frequency than the parent compound DPI 201-106.

Keywords: BDF 9148; DPI 201-106; inotropic effect; action potential; atrium; papillary muscle

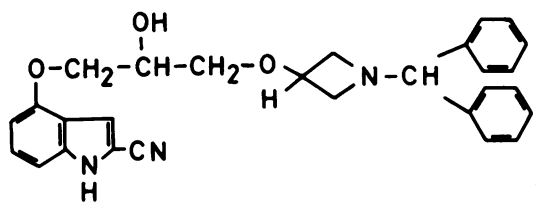
Introduction

Cardiac glycosides are effective in congestive heart failure, but their safety margin is small and toxic side-effects are not uncommon. Inhibitors of phosphodiesterase III have been tried as an alternative therapeutic principle for this disease. However, these drugs are potentially arrhythmogenic because they increase the adenosine 3′:5′-cyclic monophosphate (cyclic AMP) level of the myocardial cells. Furthermore, in the failing heart the number of β-adrenoceptors is reduced (Bristow *et al.*, 1982) and the stimulation of adenylate cyclase is impaired by an increased concentration of G_i-proteins (Feldman *et al.*, 1987). Therefore, the effectiveness of phosphodiesterase inhibitors is reduced in end-stage heart failure (Feldman *et al.*, 1987; Böhm *et al.*, 1988), and there is still a need for new positive inotropic drugs which are clinically effective and safe in congestive heart failure.

An increased intracellular sodium concentration inhibits the diastolic calcium extrusion via the sodium-calcium exchange mechanism. Therefore, drugs which increase the sodium influx may increase the contractile force. The positive inotropic effect of naturally occurring sodium channel activators cannot be exploited therapeutically, because these drugs cause a dramatic prolongation of the action potential duration, can trigger afterdepolarizations and thus induce arrhythmias (Honerjäger, 1982). However, the piperazinyl-indole DPI 201-106 has been used successfully in some recent clinical trials (Kostis *et al.*, 1987; Butrous *et al.*, 1988; Hogan *et al.*, 1988). BDF 9148, systematic name 4-[3-(1-diphenylmethyl-azetidine-3-oxy)-2-hydroxy-propoxy]-1-H-indole-2-carbonitrile, is a recently synthesized derivative, where the piperazinyl moiety of DPI 201-106 has been replaced by an azetidine-3-oxy-moiety (Figure 1). Several preliminary communications (Armah *et al.*, 1990a,b; Brasch, 1990; Brasch & Iven, 1990) have shown that this new drug increases the contractile force of heart muscle preparations *in vitro*. In the



DP I 201–106



BDF 9148

Figure 1 Chemical structure of BDF 9148 and DPI 201-106.

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present paper we compare the effects of BDF 9148 and DPI 201-106 in guinea-pig isolated atria and papillary muscles.

Methods

Experimental procedure

Male guinea pigs (300–500 g) were killed by a blow on the head. The hearts were rapidly removed and right or left atria or papillary muscles from the right ventricle were dissected in cold, oxygenated Krebs-Henseleit solution. The muscles were then mounted in 20 ml organ baths filled with Krebs-Henseleit solution of the following composition (mmol l^{-1}): NaCl 117.6, KCL 5.8, $CaCl_2$ 1.8, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and dextrose 5.5. They were kept at 32 ± 0.2°C and constantly gassed with 95% O_2 and 5% CO_2. The low temperature was chosen because at 37°C the oxygen demand is greater and many preparations show a marked decline of the contractile force during a control period of some hours. This decline is negligible at 32°C which, therefore, is a commonly used temperature for *in vitro* experiments with heart muscle. The resting tension was adjusted to 10 mN.

Right atria were allowed to beat spontaneously. Left atria and papillary muscles were stimulated at a rate of 1 Hz via platinum electrodes with square pulses of 1 ms duration and 1.5 times threshold voltage. The force of contraction was registered isometrically by a K30 force-displacement transducer (Hugo-Sachs, Hugstetten, F.R.G.) and the isometric contraction curve was displayed on a Hellige Helcoscriptor (Hellige, Freiburg, F.R.G.). The time to peak force (t_1) and the relaxation time (t_2) were measured at 10% of the maximum of the isometric contraction curve. Action potentials (AP) from papillary muscles were recorded with standard glass microelectrodes which were filled with 3 M KCl and had a tip resistance of 5–10 MΩ. The maximum depolarization velocity $\dot{V}_{max}$ was obtained by passive signal differentiation. The AP and $\dot{V}_{max}$ were displayed on a Tektronix 502 A dual-beam oscilloscope (Tektronix Inc., Beaverton, OR, U.S.A.) and photographed with a Grass C4 Camera (Grass Medical Instruments, Quincy, MA, U.S.A.) for subsequent analysis. To determine the functional refractory period (FRP), an extra stimulus was inserted with a variable time interval after every sixth regular stimulus. This extra stimulus was delivered automatically by the two channel stimulator which also produced the continuous train of driving pulses.

Experiments were started after an equilibration period of 60–90 min. Cumulative concentration-response curves for BDF 9148, DPI 201-106 or ouabain were obtained by increasing the drug concentration stepwise in 20 min or 40 min intervals. For the evaluation of action potential changes, only those experiments were included in the study in which microelectrode recordings could be obtained from the same cell during the whole time course of the experiment.

Assay of BDF 9148

The concentration of BDF 9148 in left atria was measured by high performance liquid chromatography (h.p.l.c.). The atria were removed from the organ bath immediately after the end of the experiment, blotted between filter paper, weighed and stored at −20°C. For analysis, the atria were homogenized in 1 ml isopropanol with an Ultraturrax. Four ml chloroform and 200 μl saturated bicarbonate buffer, pH 9.8, were then added and the mixture shaken for 10 min and centrifuged. Three ml of the chloroform phase was evaporated to dryness under a stream of air. The residue was redissolved in 100 μl of the mobile phase for chromatography which consisted of (v/v) methanol 13%, acetronitrile 30%, distilled water 53.5%, acetic acid 1% and tetrahydrofurane 2.5%; 20 μl was injected onto a RP Select B column (Merck, Darmstadt, F.R.G.). The mobile phase was pumped through the column at a rate of 1 ml min^{-1}. BDF 9148 was detected and quantified by measuring the absorbance at 280 nm with a Shimadzu SP-4 UV detector. Calibration curves were obtained by adding 100–1000 ng BDF 9148 to unpretreated atria.

Drugs

Racemic BDF 9148 and DPI 201-106 were kindly provided by Beiersdorf AG (Hamburg, F.R.G.). The other drugs used were ouabain (Sigma, Deisenhofen, F.R.G.), tetrodotoxin (Sankyo, Tokyo, Japan) and propranolol hydrochloride (Rhein-Pharma, Heidelberg, F.R.G.). The reagents used for h.p.l.c. were of analytical grade and were obtained from Merck (Darmstadt, F.R.G.). BDF 9148 and DPI 201-106 had to be dissolved in dimethyl sulphoxide (DMSO). Microlitre amounts of the stock solutions were added to the organ bath and at the end of a concentration-response curve of either drug, the concentration of DMSO in the bath never exceeded 0.75%. All other drugs were dissolved in double distilled water. Stock solutions of all drugs were prepared daily.

Statistics

Arithmetic means were calculated for the pre-drug values of all parameters and for the drug-induced changes. Student's t test for paired data was then used to assess the statistical significance of the observed mean changes. $P \leqslant 0.05$ for the two-tailed test was fixed as the criterion for acceptance of statistical significance throughout. For the construction of concentration-response curves, the positive inotropic effect of each concentration of BDF 9148, DPI 201-106 or ouabain was expressed as a percentage of the corresponding maximum effect. EC_{50} values were then calculated from the log concentration-effect curves after linearization by probit transformation and estimation of the regression function with the least squares method. Regression lines were compared as described by Documenta Geigy (1968).

Results

Chronotropic effect

At the beginning of the experiments, the beating frequency of right atria ranged between 160 and 170 beats per minute (b.p.m.). Increasing the concentration of BDF 9148 stepwise in 20 min intervals up to 3×10^{-6} mol l^{-1} caused no significant change of the contraction rate. Only with the final concentration of 10^{-5} mol l^{-1} a slight reduction to 144 ± 5 b.p.m. was observed (Figure 2). DPI 201-106, in concentrations between 10^{-7} mol l^{-1} and 10^{-5} mol l^{-1}, caused a concentration-dependent decrease of the beating frequency from 161 ± 5 b.p.m. to 86 ± 7 b.p.m. (Figure 2). The solvent DMSO, which was added cumulatively in 6 control experiments up to a final concentration of 0.75%, did not change the contraction rate significantly (data not shown).

Inotropic effect

Cumulative concentration-response curves (dosing interval 40 min) for the positive inotropic effect of BDF 9148 and DPI 201–106 are shown in Figure 3. In left atria, BDF 9148 increased the force of contraction from 4.3 ± 1.0 mN to 11.5 ± 1.8 mN and DPI 201-106 caused an increase from 2.5 ± 0.5 mN to 10.9 ± 0.7 mN. We cannot explain why the predrug contractile force was so much smaller in the DPI 201-106-treated muscles, but we are confident that a technical mistake in the experiments can be excluded. Due to this discrepancy, the % force increase obtained with BDF 9148 (+167%) was smaller than the % increase produced by DPI 201-106 (+336%) though the maximum developed force was nearly identical with both drugs. Also, the EC_{50} values of the two compounds were not significantly different. For BDF 9148 an EC_{50} of 1.32×10^{-7} mol l^{-1} was calculated (95%

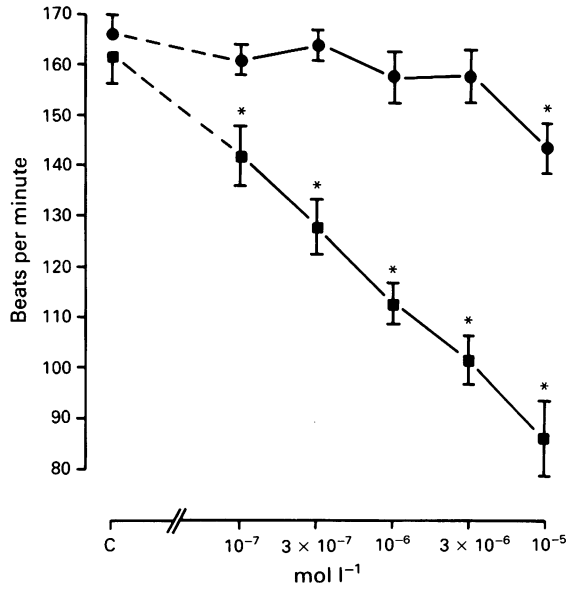

Figure 2 Influence of BDF 9148 (●) and DPI 201-106 (■) on the beating frequency of right atria. Drugs were added cumulatively in 20 min intervals. Abscissa scale: drug concentrations (mol l^{-1}). Ordinate scale: beating frequency (b.p.m.). C = pre-drug control value. Means and their standard errors (vertical bars) ($n = 10$ for BDF, $n = 6$ for DPI) are shown. * The beating frequency is significantly different from the corresponding predrug control ($P \leq 0.05$; t test for paired data).

confidence interval $0.46–3.78 \times 10^{-7}$ mol l^{-1}) and the corresponding value for DPI 201-106 was 2.6×10^{-7} mol l^{-1} ($2.0–3.4 \times 10^{-7}$ mol l^{-1}).

In papillary muscles, BDF 9148 increased the contractile force from 2.0 ± 0.6 mN to 4.4 ± 0.8 mN (+120%), and DPI 201-106 produced an increase from 2.6 ± 0.4 mN to 5.8 ± 0.8 mN (+123%). The EC_{50} for BDF 9148, however, (7×10^{-7} mol l^{-1}; 95% confidence interval $5.6–8.8 \times 10^{-7}$ mol l^{-1}) was significantly greater than for DPI 201-106 (2.8×10^{-7} mol l^{-1}; 95% confidence interval $2.2–3.5 \times 10^{-7}$ mol l^{-1}).

Both drugs also changed the shape of the isometric contraction curve. Typical examples are shown in Figure 4. The main effect was a concentration-dependent prolongation of the relaxation time of atria and papillary muscles (Table 1). In both cases, the maximum increase caused by DPI 201-106 was greater than the maximum effect of BDF 9148. The time to peak force of atria was also prolonged by both drugs while the time to peak force of papillary muscles was not affected by BDF 9148 and slightly shortened by DPI 201-106.

Interaction of BDF 9148 with other drugs

After addition of 10^{-6} mol l^{-1} BDF 9148 to the organ bath, the force of contraction of left atria increased from 4.3 ± 0.9 mN to 10.0 ± 1.2 mN within 20 min and remained nearly constant thereafter (Figure 5). A similar time course was observed for the prolongation of the relaxation time from 85 ± 6 ms to 140 ± 7 ms. After 1 h of drug exposure, the effects of BDF 9148 could not be reversed or reduced by a 30 min washout period (Figure 5).

In 6 atria, the effect of 10^{-6} mol l^{-1} BDF 9148 was tested in the presence of 4×10^{-6} mol l^{-1} propranolol. The β-blocker was added 30 min before BDF 9148 and did not attenuate the positive inotropic effect of the latter. In these experiments the contractile force increased from 2.4 ± 0.3 mN to 6.9 ± 1.0 mN. The relaxation time, however, was prolonged much less (from 80 ± 3 ms to 95 ± 4 ms) than with BDF 9148 alone.

Tetrodotoxin, 3×10^{-6} mol l^{-1}, completely reversed the positive inotropic effect of 10^{-6} mol l^{-1} BDF 9148 in atria and also the associated increase of the relaxation time (Figure

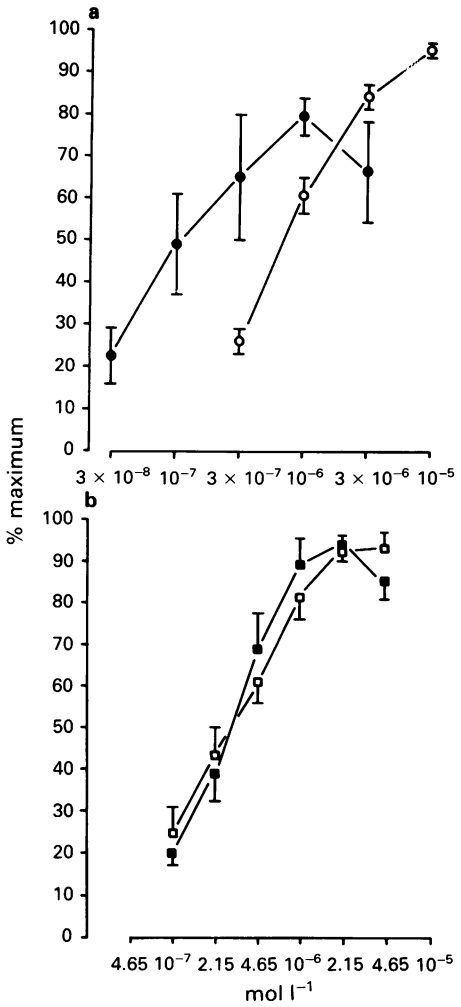

Figure 3 Cumulative concentration-response curves for the positive inotropic effect of BDF 9148 (a) and DPI 201-106 (b) in left atria (filled symbols) and papillary muscles (open symbols). Means and their standard errors ($n = 6–8$) are shown. Abscissae: drug concentration (mol l^{-1}). Ordinates: force increase as percentage of the corresponding maximum increase.

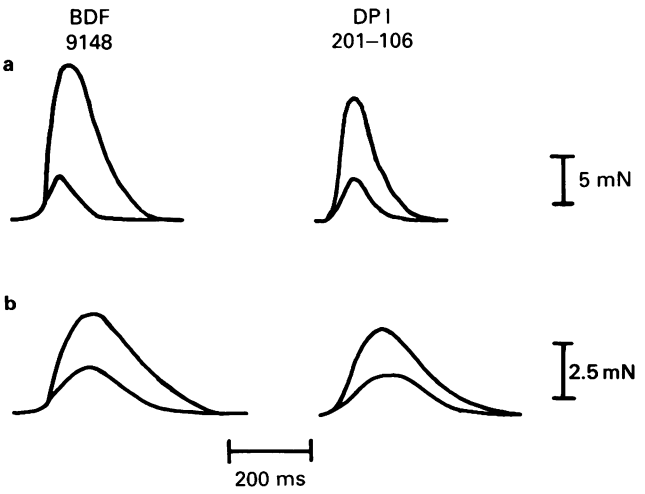

Figure 4 Typical examples of the influence of BDF 9148 and DPI 201-106 on the shape of the isometric contraction curve of left atria (a) and papillary muscles (b). Each panel shows the pre-drug control curve and the effect of either BDF 9148 (10^{-6} mol l^{-1} in the atrium and 10^{-5} mol l^{-1} in the papillary muscle) or DPI 201-106 (10^{-6} mol l^{-1} in both cases).

Table 1 Influence of BDF 9148 and DPI 201-106 on the time to peak force (t_1) and the relaxation time (t_2) of left atria and papillary muscles

	Left atrium		*Papillary muscle*	
Drug	t_1 (ms)	t_2 (ms)	t_1 (ms)	t_2 (ms)
Control	48 ± 1.1	189 ± 2.7	111 ± 8.9	164 ± 13.5
BDF 9148 (mol l^{-1})				
3×10^{-8}	+0.8 ± 1.5	+7.5 ± 3.8	NT	NT
10^{-7}	+3.3 ± 2.1	+15.8 ± 5.8*	NT	NT
3×10^{-7}	+6.7 ± 1.1*	+30.8 ± 6.2*	−0.6 ± 6.6	+20.6 ± 5.0*
10^{-6}	+10.8 ± 1.5*	+39.1 ± 4.4*	+1.9 ± 5.8	+39.4 ± 7.5*
3×10^{-6}	+9.1 ± 2.0*	+40.0 ± 2.9	+6.9 ± 4.8	+51.9 ± 11.2*
10^{-5}	NT	NT	+3.1 ± 6.0	+48.1 ± 7.1*
Control	49 ± 0.8	87 ± 3.3	140 ± 6.8	171 ± 9.1
DPI 201-106 (mol l^{-1})				
10^{-7}	+3.3 ± 3.1	+12.5 ± 5.7	+2.1 ± 3.2	+3.6 ± 9.7
2.15×10^{-7}	+1.6 ± 2.1	+15.8 ± 4.4*	−4.3 ± 4.8	+21.4 ± 11.2
4.65×10^{-7}	+3.3 ± 2.1	+32.5 ± 5.4*	−4.3 ± 5.2	+45.0 ± 7.6*
10^{-6}	+9.2 ± 3.9	+56.7 ± 2.5*	−10.7 ± 4.9	+79.3 ± 11.7*
2.15×10^{-6}	+14.2 ± 1.5*	+58.3 ± 6.0*	−12.1 ± 3.6*	+98.6 ± 12.2*
4.65×10^{-6}	+13.3 ± 1.7*	+51.7 ± 6.4*	−17.8 ± 3.2*	+114.3 ± 12.9*

The data were obtained from the cumulative concentration-response curves. Pre-drug values (Control) and their mean changes 40 min after addition of each drug concentration are given ($\bar{x}$ ± s.e.mean; n = 6–8). Asterisks indicate changes that are statistically significant ($P \leq 0.05$; t test for paired data). NT = not tested.

6). In unpretreated muscles (3 preliminary experiments) the concentration of tetrodotoxin used caused a modest (about 20%) reduction of the contractile force and did not change the shape of the isometric contraction curve. The effects of BDF 9148 were reversed within minutes after addition of tetrodotoxin.

However, when both drugs were removed from the organ bath by washout, the force of contraction and the relaxation time started to increase again and reached final values that were similar to the maximum effects observed in the presence of BDF 9148 (Figure 6).

A small concentration of BDF 9148, 3×10^{-8} mol l^{-1}, which by itself caused only a 32% increase of the contractile force, shifted the concentration-response curve for the positive inotropic effect of ouabain in atria to the left (Figure 7). In unpretreated muscles, ouabain increased the force of contraction from 2.7 ± 0.2 mN to 12.4 ± 1.3 mN and the EC_{50} was 3.21×10^{-7} mol l^{-1} (95% confidence interval 2.86–3.61×10^{-7} mol l^{-1}). After preincubation with BDF 9148 for 1 h, the maximum effect of ouabain was not affected, i.e. the force increased from 4.71 ± 0.9 mN to 12.9 ± 1.7 mN, but the EC_{50} was significantly reduced to 2×10^{-7} mol l^{-1} (95% confidence interval 1.71–2.34×10^{-7} mol l^{-1}).

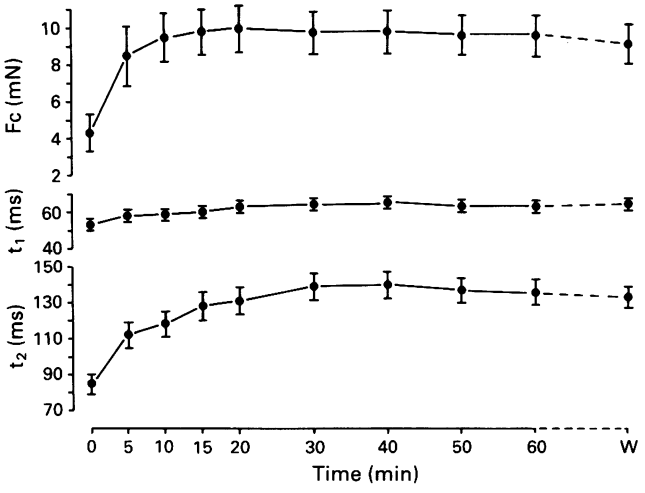

Figure 5 Influence of 10^{-6} mol l^{-1} BDF 9148 on the force of contraction (FC), time to peak force (t_1) and relaxation time (t_2) of left atria. The symbols indicate means and standard errors are shown by vertical bars (n = 6). Abscissa scale: time after drug addition (min). W = effect after 30 min washout.

Tissue concentration of BDF 9148

Two min after the addition of 10^{-6} mol l^{-1} BDF 9148 to the organ bath, the mean concentration in atria was already 15.3 ± 3.58 × 10^{-6} mol kg^{-1}, indicating a 15.3 fold accumulation of the drug in the tissue (Table 2). A prolongation of the incubation time up to 30 min did not increase the tissue concentration of BDF 9148 further. Frequent renewal of the bathing fluid during a 30 min washout period did not reduce the amount of BDF 9148 in the atria significantly; even after 5 h of washout, considerable tissue concentrations of the drug were found.

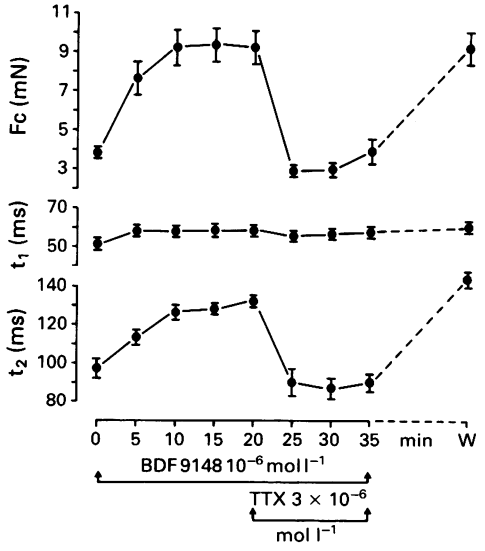

Figure 6 Influence of 10^{-6} mol l^{-1} BDF 9148 on the force of contraction (FC), time to peak force (t_1) and relaxation time (t_2) of left atria as it is antagonized by 3×10^{-6} mol l^{-1} tetrodotoxin (TTX). The symbols indicate means and standard errors are shown by vertical bars (n = 6). Abscissae: time after addition of BDF 9148 (min). The drugs were present in the organ bath as indicated by the horizontal bars below the abscissa scale. W = effect after washout (30 min) of both drugs.

Table 2 Concentration of BDF 9148 (μmol kg^{-1}) in left atria after incubation with the drug for 2–30 min and subsequent washout

	Drug concentration (μmol kg^{-1})		
Incubation time	*Incubation only*	*Incubation +30 min washout*	*Incubation +5 h washout*
2 min	15.30 ± 3.58	9.36 ± 0.64	NT
5 min	7.69 ± 1.70	10.54 ± 3.25	6.25 ± 1.57
30 min	18.80 ± 1.63	19.29 ± 1.24	NT

Means and their standard errors (n = 6) are given. NT = not tested.

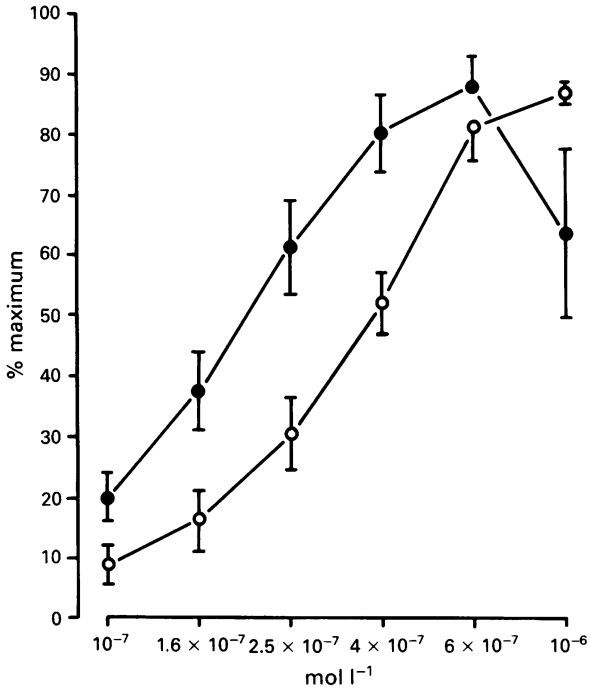

Figure 7 Cumulative concentration-response curves for the inotropic effect of ouabain in left atria: (○) ouabain alone; (●) ouabain after pretreatment with 3 × 10^{-8} mol l^{-1} BDF 9148. The symbols indicate means and standard errors are shown by vertical bars (n = 6). Abscissa scale: concentration of ouabain (mol l^{-1}). Ordinate scale: inotropic effect as a percentage of the corresponding maximum effect.

Effects on action potentials

BDF 9148 and DPI 201-106 increased the duration of action potentials from papillary muscles. Typical examples for this effect are shown in Figure 8. Table 3 lists the detailed results. The concentration of BDF 9148 was increased cumulatively from 3 × 10^{-7} mol l^{-1} to 10^{-5} mol l^{-1} in 20 min intervals and the last concentration stayed in contact with the muscle for 1 h. There were no changes of the resting potential, the amplitude or $\dot{V}_{max}$ of the action potential. The action potential duration was increased concentration-dependently at all stages of repolarization and the functional refractory period was increased to a similar extent. The effect of BDF 9148 could not be reversed by washout. With DPI 201-106, two successive concentrations were tested which were allowed to act on the muscle for 1 h each. The action potential duration and the functional refractory period were prolonged to the same extent as with equimolar concentrations of BDF 9148. The solvent DMSO, which was added cumulatively in 6 control experiments up to a final concentration of 0.75%, caused no significant change of the action potential parameters (data not shown).

In an additional series of experiments, 10^{-5} mol l^{-1} BDF 9148 was added to papillary muscles and the effect recorded after 30 min. In this case the prolongation of the action potential duration was much smaller than in the experiments described above (+16 ± 2.6 ms versus +54 ± 6.1 ms, see Table 3). In the single concentration experiments, the total duration of the BDF 9148 exposure was shorter than in the cumulative concentration-response experiments (30 min versus at least 80 min), but admittedly this is not a really satisfying explanation for the variability of the drug effect. When

Table 3 Influence of BDF 9148, DPI 201-106 and tetrodotoxin (TTX) on action potential parameters of papillary muscles. Drugs were added cumulatively

		MRP (mV)	*APA* (mV)	$\dot{V}_{max}$ (V s^{-1})	APD_{20} (ms)	APD_{80} (ms)	*FRP* (ms)
Control		82 ± 1.9	111 ± 0.9	152 ± 5	131 ± 5	220 ± 8	229 ± 9.0
BDF 9148 (mol l^{-1})							
3 × 10^{-7}	Δ20 min	−0.3 ± 0.3	−0.8 ± 0.5	+1.2 ± 2.7	+2.0 ± 2.5	+6.5 ± 2.3*	+5.0 ± 2.2
10^{-6}	Δ20 min	+0.7 ± 0.5	−0.5 ± 0.8	+4.7 ± 1.3*	+12.2 ± 3.5*	+15.5 ± 4.7*	+21 ± 5.5*
3 × 10^{-6}	Δ20 min	+0.5 ± 0.3	+0.3 ± 0.7	−0.2 ± 8.9	+24.3 ± 5.6*	+33.3 ± 5.8*	+36 ± 7.5*
10^{-5}	Δ20 min	+0.8 ± 0.5	+0.5 ± 1.4	+2.5 ± 5.3	+40.2 ± 6.8*	+54.0 ± 6.1*	+55 ± 8.7*
10^{-5}	Δ60 min	+1.0 ± 0.5	+0.5 ± 0.9	−0.3 ± 7.4	+40.2 ± 6.3*	+58.5 ± 5.2*	+63 ± 7.6*
Washout	Δ30 min	−0.3 ± 0.4	−1.8 ± 1.3	+4.6 ± 5.7	+30.5 ± 5.3*	+54.0 ± 6.6*	+60 ± 7.1*
Control		79 ± 0.6	115 ± 1.0	149 ± 7.3	141 ± 7	214 ± 12	228 ± 13.8
DPI 201-106							
3 × 10^{-6}	Δ20 min	+0.6 ± 1.2	+0.3 ± 0.5	−2.1 ± 4.7	+14.7 ± 3.9*	+16.3 ± 4.1*	+15.7 ± 3.3*
3 × 10^{-6}	Δ60 min	+0.3 ± 1.6	0.0 ± 0.4	+9.7 ± 6.2	+22.1 ± 6.2*	+31.0 ± 6.8*	+33.6 ± 8.1*
10^{-5}	Δ20 min	+0.4 ± 1.6	−0.8 ± 0.8	+0.6 ± 6.6	+20.8 ± 7.7*	+46.0 ± 12.4*	+58.7 ± 12.0*
10^{-5}	Δ60 min	+1.3 ± 1.2	−0.6 ± 0.6	−1.0 ± 4.6	+30.0 ± 6.5*	+61.0 ± 12.4*	+63.6 ± 13.5*
Control		79 ± 2.1	113 ± 1.5	147 ± 6.2	146 ± 5.8	245 ± 12.7	258 ± 16.6
BDF 10^{-5}	Δ30 min	+0.8 ± 0.9	+1.0 ± 0.9	+0.3 ± 1.3	14.7 ± 3.7*	+16.0 ± 2.6*	+25.0 ± 1.3*
+TTX 3 × 10^{-6}	Δ10 min	+1.3 ± 0.7	+0.7 ± 1.6	−32.8 ± 5.0*	−6.7 ± 3.1	−24.5 ± 4.4*	0.0 ± 8.2
Washout	Δ30 min	−0.3 ± 1.0	0.0 ± 2.5	−7.0 ± 6.1	+20.5 ± 6.4	+27.5 ± 6.2*	+37.5 ± 7.4*

The pre-drug values (Control) and their mean changes (Δ) 10, 20, 30 or 60 min after addition of each drug concentration are shown ($\bar{x}$ ± s.e.mean; n = 6). Asterisks indicate changes that are statistically significant ($P \leq 0.05$; t test for paired data). Abbreviations: MRP = membrane resting potential; APA = action potential amplitude; $\dot{V}_{max}$ = maximum depolarization velocity; APD_{20}, APD_{80} = action potential duration at 20% and 80% of full repolarization; FRP = functional refractory period.

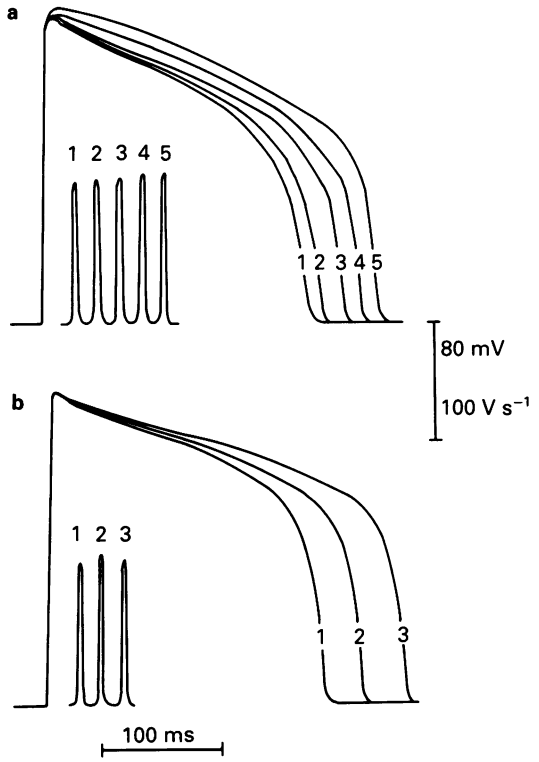

Figure 8 Typical examples for the influence of BDF 9148 and DPI 201-106 on the action potential of papillary muscles (cumulative drug dosing). (a) Control action potential AP (1) and effect of 3×10^{-7} mol l^{-1} (2), 10^{-6} mol l^{-1} (3), 3×10^{-6} mol l^{-1} (4) and 10^{-5} mol l^{-1} (5) BDF 9148 after 20 min exposure to each drug concentration. Although there was a small increase of the action potential amplitude with 10^{-5} mol l^{-1} in this experiment, the mean effect of BDF 9148 on this parameter was not statistically significant (see Table 3). (b) Control AP (1) and effect of 3×10^{-6} mol l^{-1} (2) and 10^{-5} mol l^{-1} (3) DPI 201-106 after 60 min exposure to each drug concentration. The corresponding $\dot{V}_{max}$ values are given in the insets.

3×10^{-6} mol l^{-1} tetrodotoxin was added 30 min after 10^{-5} mol l^{-1} BDF 9148, the influence of the latter was promptly reversed (Table 3). Tetrodotoxin also caused a significant decrease of $\dot{V}_{max}$. After washout of both drugs, $\dot{V}_{max}$ was restored and action potential duration and functional refractory period increased again to values slightly above the maximum that was observed in the presence of BDF 9148 beforehand.

Discussion

The positive inotropic effect of the new drug BDF 9148 is not very different from the effect of the parent compound DPI 201-106. Both drugs increased the contractile force of papillary muscles to nearly the same extent. That in atria BDF 9148 produced a much smaller percentage increase of the force than DPI 201-106 should not be overemphasized because the corresponding predrug values were quite different. The maximum force obtained with either compound was nearly identical and the two EC_{50} values were not significantly different in this tissue. But while for DPI 201-106 the EC_{50} was the same in atria and papillary muscles, the sensitivity of the two tissues towards BDF 9148 was different, the EC_{50} being about five fold greater in papillary muscles than in atria. The explanation for this finding is not known.

The inotropic effect of BDF 9148 was not prevented by the β-blocker propranolol. Thus, it cannot be due to a direct stimulation of β-adrenoceptors or to a release of endogenous catecholamines. The associated prolongation of the relaxation time makes it unlikely that the positive inotropic effect is mediated by an increase of the intracellular cyclic AMP level. A slowing of the relaxation process was also seen with DPI 201-106 and, in general, is a typical finding with drugs which prolong the open state of the sodium channel (Honerjäger, 1982). As the effect of BDF 9148 was promptly reversed by the sodium channel inactivator tetrodotoxin, an increased sodium influx seems to be the most likely explanation for the drug-induced increase of contractility. Recent measurements of the sodium current in rat isolated myocytes have confirmed this mechanism of action (Honerjäger *et al.*, 1990). The leftward shift of the concentration-response curve of ouabain produced by BDF 9148 fits into the picture because an inhibition of the Na^+-K^+-ATPase should have a greater effect when the sodium load of the cells is increased. Similar interactions with cardiac glycosides have been observed with DPI 201-106 (Scholtysik *et al.*, 1989) and veratridine (Honerjäger & Reiter 1975).

BDF 9148 and DPI 201-106 prolonged the action potential duration at all stages of repolarization. For DPI 201-106, this is a well known effect (Buggisch *et al.*, 1985; Scholtysik *et al.*, 1985; Kohlhardt *et al.*, 1987; Kihara *et al.*, 1989) which is best explained by a slowed inactivation of the inward sodium current. The increase in action potential duration probably accounts for the associated prolongation of the functional refractory period. In comparison to some ceveratrum alkaloids, which cause an excessive prolongation of the terminal repolarizaton phase (Honerjäger, 1982), the overall effect of BDF 9148 and DPI 201-106 on the action potential duration is small. Wang *et al.* (1990) have shown that the enantiomers of DPI 201-106 have opposite effects on the sodium channel. (S)-DPI increases the peak inward current and slows its inactivation while (R)-DPI reduces the peak current. Thus the maximum effect of racemic DPI, which was used in our experiments, is limited by a kind of auto-inhibitory mechanism, which may explain the low arrhythmogenicity of this drug. A similar mechanism could limit the action potential prolongation by racemic BDF 9148 (Honerjäger *et al.*, 1990).

BDF 9148 is very lipophilic and only sparingly soluble in water. In aqueous solution, the maximum concentration that can be achieved is about 3×10^{-5} mol l^{-1}. Due to its physicochemical properties, the drug is quickly accumulated in heart tissues. Steady-state tissue concentrations were established within a few minutes. The pharmacological effects of the drug, on the other hand, took much longer to develop. This may indicate that the overall tissue concentration does not reflect the concentration at the pharmacologically relevant binding site in the sodium channel. It was almost impossible to remove BDF 9148 from the atria by washout, which explains the unusual persistence of the drug effects. Effects of BDF 9148 that had been reversed by tetrodotoxin reappeared promptly when both drugs were removed from the bathing fluid, obviously because the water soluble tetrodotoxin was washed away from the sodium channel while the lipophilic BDF 9148 persisted. A similar observation has been made with DPI 201-106 (Buggisch *et al.*, 1985), which is also very lipophilic.

There was one major difference between BDF 9148 and DPI 201-106: while the former had only a small effect on the beating frequency of right atria, a concentration-dependent negative chronotropic effect was seen with DPI 201-106. This drug binds to vascular (Hof & Hof, 1985) and cardiac (Holck & Osterrieder, 1988; Siegl *et al.*, 1988) calcium channels and reduces the slow inward current in slow response action potentials (Buggisch *et al.*, 1985). Thus, calcium antagonism is a probable explanation for the negative chronotropic effect of DPI 201-106. It may also be the cause of the slight reduction of the atrial rate that was observed with the higher concentration of BDF 9148 in our experiments. The finding that beyond 10^{-7} mol l^{-1}, BDF 9148 reduces the calcium current in myocytes (Pfeifer & Ravens, 1990) points in this direction. If this explanation is accepted, a comparison of the negative chronotropic effects of DPI 201-106 and BDF 9148 suggests that the calcium antagonistic properties of the latter are much

weaker than those of the parent compound. However, before a detailed comparison of the influence of both drugs on the cardiac calcium channels has been made, such an assumption remains speculative.

We observed only an increase of the action potential duration in the presence of BDF 9148, while Armah *et al.* (1990a,b) have described a biphasic effect, i.e. a transient prolongation followed by a gradual shortening. The discrepancy may be due, at least in part, to methodological differences. While we increased the concentration of BDF 9148 at 20 min intervals, Armah *et al.* (1990a,b) observed the effect of a single concentration for 90 min. In their experiments action potential shortening usually began after 30–60 min of drug exposure. Thus, even the 1 h observation period with 10^{-5} mol l^{-1} BDF 9148 in our experiments may have been too short for the development of a biphasic drug effect. However, if the increase of the action potential duration is indeed transient *in vivo*, this could explain why no increase of the Q–T interval of the ECG is seen in the presence of BDF 9148 (Muster & Raap 1990) while DPI 201-106 usually causes a prolongation (Ruegg & Nuesch, 1987; Kostis *et al.*, 1987).

To sum up, BDF 9148 is an ionotropic compound structurally related to DPI 201-106. Our results confirm and extend earlier findings which support the hypothesis that both drugs increase the contractile force via a prolongation of the open state of the sodium channel. BDF 9148 differs from its parent compound mainly by a much weaker influence on the spontaneous beating frequency. The drug may be beneficial in the treatment of congestive heart failure and its *in vitro* and *in vivo* effects certainly deserve further investigation.

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Measurements of tacrine and monoamines in brain by *in vivo* microdialysis argue against release of monoamines by tacrine at therapeutic doses

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1 The concentration of tacrine (tetrahydroaminoacridine or THA) in plasma, regions of brain and cerebral extracellular fluid has been studied in the rat at various times following injection of a dose of 5 mg kg^{-1}, i.p.

2 The peak plasma THA concentration was 2.46 nmol ml^{-1}, and occurred 30 min post injection and clearance was first order ($t_{1/2}$ = 90 min). The concentration in the brain peaked between 30–60 min, and was around 30 times plasma concentration (striatum peak concentration = 65 ± 3 nmol g^{-1}). Extracellular cerebral concentration measured by *in vivo* microdialysis was similar to plasma concentration with the peak occurring 100 min post-injection.

3 No evidence was obtained by *in vivo* dialysis for THA inducing dopamine release from striatum or 5-hydroxytryptamine (5-HT) release from the frontal cortex. Enhanced release of dopamine did occur after (+)-amphetamine (5 mg kg^{-1}, i.p.) injection, while KCl (100 mM) in the probe released both dopamine and 5-HT.

4 Since the minimum plasma THA concentration achieved in this study was at least twice that found in the plasma of patients given THA for the treatment of dementia, these results suggest that monoamine release in the brain does not occur during therapy.

Keywords: Tacrine; *in vivo* microdialysis; monoamine release *in vivo*; 5-hydroxytryptamine release; dopamine release; cholinesterase inhibition; tacrine drug-distribution; dementia

Introduction

Tacrine (tetrahydroaminoacridine or THA) is a cholinesterase inhibitor that has been reported to be of therapeutic benefit to some patients with clinically diagnosed Alzheimer-type dementia (see for example Summers *et al.*, 1986). Whilst several other cholinesterase inhibitors and cholinoceptor agonists have been investigated in senile dementia, none has been found to be as effective as THA (for review see Whalley, 1989). This has led to the suggestion that THA may possess other pharmacological properties of benefit in dementia.

In view of the structural similarity of THA to 4-aminopyridine, a potassium channel antagonist, Summers *et al.* (1986) suggested that THA might block these channels and there is some electrophysiological evidence for this (Drukarch *et al.*, 1987; Osterreider, 1987; Rogawski, 1987; Schauf & Sattin, 1987; Stevens & Cotman, 1987) albeit only at high concentrations of THA (0.1–1 mM).

Studies *in vitro* on rat brain tissue slices (Robinson *et al.*, 1989) have shown that THA can induce the release of endogenous 5-hydroxtryptamine (5-HT) and dopamine and do so by a mechanism that does not require cholinergic mechanisms since cholinoceptor blockade does not affect the response. Whilst the concentrations of THA required to enhance monoamine release are lower than those required to block potassium channels, it is not clear whether such an effect occurs *in vivo* following doses of THA necessary to inhibit acetylcholinesterase. In the present study the technique of intracerebral dialysis has been used to examine 5-HT and dopamine release from rat brain *in vivo*.

Initial experiments examined the concentration of THA in both plasma and brain to ensure that doses given were sufficient to achieve concentrations as least as great as those seen in plasma during clinical studies.

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Methods

Animals

Male Lister hooded rats (Harlan Olac, Bicester, U.K.) weighing 250–400 g were housed in groups of 6 in conditions of constant temperature (21°C) and controlled lighting (light period 07 h 00 min–19 h 00 min) and fed a diet of RMI expanded pellets and tap water *ad libitum*.

Determination of tacrine concentration in plasma, brain and cerebral extracellular fluid

THA was determined in plasma, brain and cerebral extracellular fluid at various times after injection of THA (5 mg kg^{-1}, i.p.).

To determine the drug in plasma or brain regions, animals were killed and blood collected or the brain removed.

Plasma was diluted 1:4 in perchloric acid (final concentration 0.3 M) and centrifuged at 10,000 *g* for 15 min. The supernatant was neutralised with K_2CO_3 (5 M) and stored overnight on ice. Samples were then centrifuged at 10,000 *g* for 15 min and the supernatant taken for determination of THA.

The brain was dissected and cortex, striatum and cerebellum isolated. Regions were homogenized in 0.4 M perchloric acid, centrifuged at 10,000 *g* for 5 min and the supernatant stored at −20°C prior to analysis.

For studies using intracerebral dialysis, probes were implanted in the striatum (see below) 24 h before drug administration. At the start of the experiment animals were anaesthetized with chloral hydrate (350 mg kg^{-1}, i.p.) and anaesthesia maintained through sample collection. The perfusate (artificial CSF, composition mM: NaCl 125, KCl 2.5, $MgCl_2$ 1.18, $CaCl_2$ 1.26) was pumped at a rate of 1 μl min^{-1} and 20 or 40 μl fractions collected. The efficacy of each dialysis probe was determined before implantation by perfusion in standard solutions of THA in artificial CSF.

THA concentrations were determined by a modification of the high performance liquid chromatography (h.p.l.c.) method of Forsyth *et al.* (1988). Dialysis samples, plasma extracts and tissue extracts were injected directly into a 250 × 4.6 mm Hypersil ODS (5 μm) column with a Spheri-5 RP18 guard column, and eluted with 33% methanol in H_2O containing 1% triethylamine pH 5.0 at a flow rate of 1.0 ml min^{-1}. Peaks were detected with a fluorescence detector (Spectrovision, U.K.) excitatory wavelength 254 nm, emission wavelength 375 nm.

Monoamine release in vivo

Basically two protocols were used. The first involved implantation of a dialysis probe into the striatum in order to examine dopamine release, which was measured in anaesthetized animals since perfusion with a high K^+ concentration (which was used to confirm the viability of the release mechanisms) led to behavioural stimulation. The second protocol examined the release of 5-HT from the frontal cortex which was performed in freely moving rats as behavioural stimulation was not observed following perfusion with a high K^+ concentration.

Implantation of dialysis probes

Rats were anaesthetized using sodium pentobarbitone (Sagatal, 60 mg kg^{-1} i.p.) and secured in a Kopf stereotaxic frame with the tooth-bar at −3.3 mm below interaural zero. In the dopamine-release and THA concentration studies rats were implanted with a 4.0 mm dialysis probe into the striatum: +0.8 mm anterior to bregma, ±3.0 mm lateral and −7.0 mm below the surface of the brain. In the 5-HT release studies rats were implanted with a 5.0 mm dialysis probe into the frontal cortex: +3.2 mm anterior to bregma, ±2.0 mm lateral and −5.8 mm below the surface of the brain. Concentric dialysis probes (diameter, 200 μm) essentially as described by Hutson *et al.* (1985) were used except that they were implanted without the use of a guide assembly and the internal glass capillary tubes were replaced by fused silica tubes.

The probes were secured to the skull with 2 screws and dental cement. Two pieces of plastic tubing (Portex pp10) were attached to the inlet and outlet tubes of the dialysis probe and secured in place with quick-setting glue. The rats were then placed into individual cages and left to recover for approximately 20 h.

Collection of dialysate samples

In the investigation on dopamine release, rats were anaesthetized with chloral hydrate (350 mg kg^{-1}, i.p.) and anaesthesia maintained throughout sample collection. However in the study on 5-HT release, rats were placed in 30 × 30 × 25 cm Perspex cages where they were allowed to move freely during sample collection. Dialysis probes were perfused with artificial CSF at a rate of 1 μl min^{-1}. The perfusate also contained citalopram (1 μM) when 5-HT release was being investigated.

After a 1–2 h equilibration period, successive 30 or 60 min samples were collected into tubes containing 30 or 60 μl, respectively, perchloric acid (0.01 M) and cysteine hydrochloride (0.2%). After collection of two baseline samples, animals received an injection of either THA (5 mg kg^{-1}) or vehicle. One hour later the concentration of KCl in the perfusate was increased to 100 mM for 30 min in half of the animals in each treatment group. Probes were then perfused with normal CSF for another 90 min. All dialysis samples from the study examining 5-HT release were analysed immediately. Samples from the study on dopamine release were frozen in solid CO_2 until analysis. Dopamine and 5-HT were stable in these conditions.

Determination of dialysate dopamine and 5-hydroxytryptamine concentrations

Dialysis samples were analysed for either dopamine or 5-HT by h.p.l.c. with coulometric detection as described previously (Robinson *et al.*, 1989). The minimum levels of detection were 20 pg for dopamine and 5 pg for 5-HT.

Behavioural observations

After injection of THA or vehicle, animals were observed for signs of chewing, flat body posture and tremor.

Drugs

Drugs were obtained from the following sources (in parentheses): 9-amino-1,2,3,4-tetrahydroacridine HCl hydrate (THA) (Aldrich Chemical Co., Dorset); sodium pentobarbitone (Sagatal) (May and Baker Ltd., Dagenham); chloral hydrate and (+)-amphetamine (Sigma Chemical Co., Dorset). Citalopram was a gift from Lundbeck, Copenhagen, Denmark, THA was dissolved in a vehicle of dimethylsulphoxide (20% v/v) and saline. All drug concentrations refer to free base.

Statistical analyses

Concentrations of dopamine and 5-HT have been expressed as pg/30 min dialysate sample. The dialysate concentrations of the first two samples from each rat were averaged to give a baseline value. The effect of drug treatments was investigated by two-way analysis of variance (ANOVA) with drug treatment as the between subjects factor and time as the repeated measure. The effects of THA and amphetamine were analysed separately. Individual means were compared by Newman Keul's post-hoc tests.

Results

The concentration of tacrine in plasma following its administration

In initial experiments rats were given THA (5 mg kg^{-1}) and the concentration measured in plasma at various times thereafter. The THA concentration peaked by 30 min and then declined and analysis of clearance revealed a first order decline with a $t_{1/2}$ of 90 min (Figure 1). THA concentration had reached around 2 nmol ml^{-1} by 15 min and peaked at 2.46 nmol ml^{-1} (Figure 1) while at 240 min it was at least twice the maximum plasma concentration of the drug seen in several clinical studies (see Discussion). The dose of 5 mg kg^{-1} was therefore chosen for subsequent experiments since it produced drug concentrations that were significantly greater than those seen clinically and thus sufficient to reveal if THA would be likely to have effects on monoamine release when used therapeutically.

The distribution of tacrine in the brain

Following THA (5 mg kg^{-1}) administration the drug could be readily detected in regions of rat brain and dialysate samples. Peak tissue concentrations occurred 30–60 min post-injection and were in the range 50–70 nmol g^{-1} (Figure 2). The decline was clearly not first order; however, there were insufficient data to allow further analysis.

In contrast, the extracellular concentration of the drug did not peak until around 100 min and its clearance was similar to plasma ($t_{1/2}$ = 75 min). The extracellular concentration was also some 30 times lower than the tissue concentration (Figure 3).

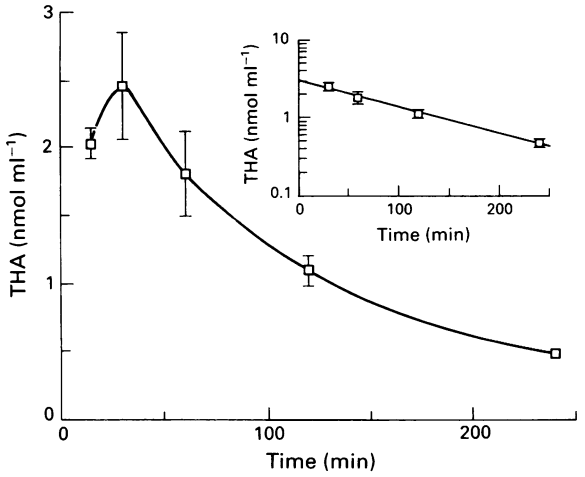

Figure 1 Plasma tacrine (THA) concentration in the 240 min following injection of THA (5 mg kg^{-1}). Results shown as mean with s.e.mean shown by vertical bars ($n = 4$). Insert graph shows log concentration versus time curve; $t_{1/2}$ of the decline = 90 min.

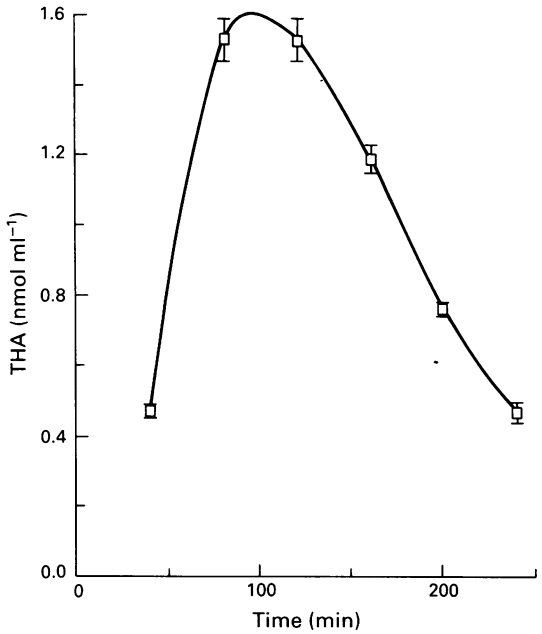

Figure 3 Extracellular tacrine (THA) concentration in rat brain following injection of THA (5 mg kg^{-1}). Results shown as mean with s.e.mean indicated by vertical bars ($n = 4$).

Effect of tacrine on dopamine release in striatum

Administration of THA (5 mg kg^{-1}) had no effect on dopamine release in the striatum as measured by *in vivo* dialysis (Figure 4a). However amphetamine (5 mg kg^{-1}, i.p.) significantly increased dialysate dopamine concentrations compared with baseline concentrations (ANOVA $F(2,6) = 4.0$; $P < 0.05$) and the effect was similar in both the THA and saline pretreated groups (Figure 4a).

Perfusion of a high K^+ solution (100 mM KCl in artificial CSF) in animals pretreated with either saline or THA (5 mg kg^{-1}) markedly enhanced dopamine release (ANOVA $F(5,60) = 12.5$; $P < 0.0001$) (Figure 4b) and the magnitude of this response was not altered by pretreatment with THA compared with saline.

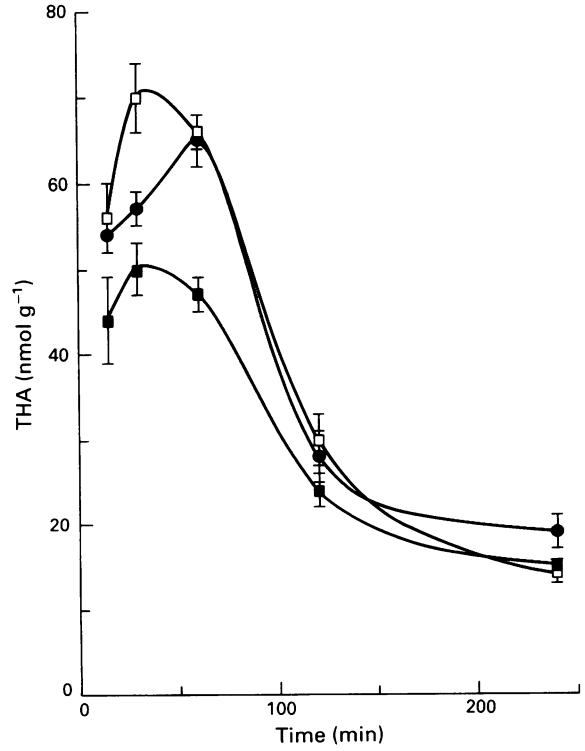

Figure 2 Concentration of tacrine (THA) in regions of rat brain following injection of THA (5 mg kg^{-1}). Mean concentration (with s.e.mean shown by vertical bars) ($n = 4$) shown in striatum (●), cortex (□) and cerebellum (■).

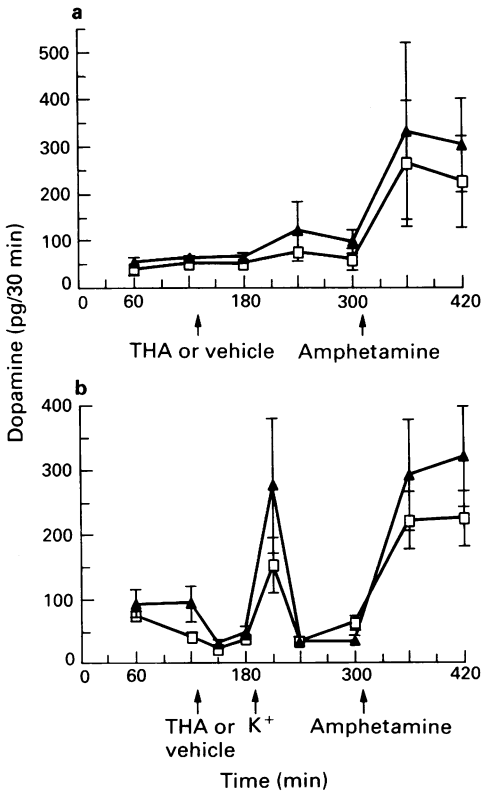

Figure 4 (a) Dialysate dopamine concentration following injection of tacrine (THA, 5 mg kg^{-1}) (□) or vehicle (▲) and (+)-amphetamine (5 mg kg^{-1}). Results shown as mean with s.e.mean indicated by vertical bars ($n = 3$ for both groups). (b) Dialysate dopamine concentration following injection of THA (5 mg kg^{-1}) (□) or vehicle (▲) and (+)-amphetamine (5 mg kg^{-1}) and addition of KCl (100 mM) to the artificial CSF. Results shown as mean with s.e.mean indicated by vertical bars. (THA-treated, $n = 8$; vehicle, $n = 6$).

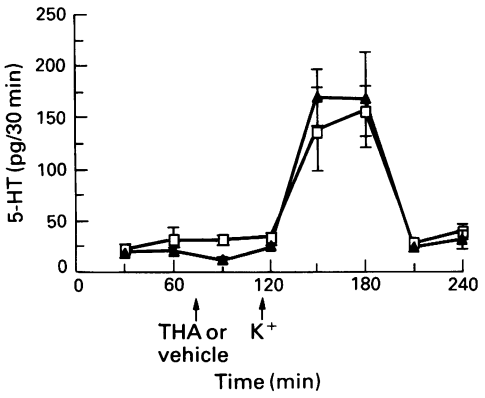

Figure 5 Dialysate 5-hydroxytryptamine (5-HT) concentration following injection of tacrine (THA, 5 mg kg^{-1}) (□) or vehicle (▲) and addition of KCl (100 mM) to the artificial CSF. Results shown as mean with s.e.mean indicated by vertical bars (n = 5 for both groups).

Effect of tacrine on 5-hydroxytryptamine release

Administration of THA (5 mg kg^{-1}) had no effect on 5-HT release from the frontal cortex either 30 min immediately following injection (Figure 5) or over a period of 2 h (data not shown). However, perfusion of CSF containing high K^+ (100 mM) markedly increased 5-HT release (ANOVA $F(5,40) = 20.1$; $P < 0.001$) and the response was similar in both THA and saline pretreated rats (Figure 5).

Behavioural observations

All the conscious animals injected with THA (5 mg kg^{-1}) showed chewing, flat body posture and tremor. These responses were seen within 5 min of administration of THA and remained constant over the next 2 h. Some tremor could still be observed 4 h later. Responses observed were about half the maximal occurring after a dose of 20 mg kg^{-1} as has previously been reported by Hunter *et al.* (1989). None of the saline-injected rats displayed any of these behaviours.

Discussion

A dose of THA (5 mg kg^{-1}, i.p.) to rats produced a concentration of the drug in the plasma at 240 min that was at least twice the steady state concentration seen in the clinical study of Summers *et al.* (1986) or the peak values seen following a dose of THA of 50 mg given to patients in the studies of Nybäck *et al.* (1988) and Forsyth *et al.* (1988). All these workers recorded plasma levels of around 50 ng ml^{-1} (= 0.25 nmol ml^{-1}). A dose of THA of 5 mg kg^{-1} in rats therefore seemed suitable in order to study what concentration of drug might occur in cerebral tissues and whether it would enhance monoamine release in the brain *in vivo* as has been observed *in vitro* (Robinson *et al.*, 1989; Drukarch *et al.*, 1988). This in turn would indicate whether such a mechanism might occur when the drug was being given to Alzheimer's disease patients.

As has been observed previously (Nielsen *et al.*, 1989) THA is concentrated in cerebral tissue following peripheral administration. The concentration in the striatum and cortex being some 25–35 times greater than in plasma 60 min after injection (compare Figures 1 and 2). The tissue concentration of THA at 2 h was also considerably higher (around 25 nmol g^{-1}) than that found by Tachiki *et al.* (1988) who reported a maximum value of 1142 ng g^{-1} (= 5.8 nmol g^{-1}) after a dose of THA of 3 mg kg^{-1}. In contrast, the cerebral extracellular THA concentration as measured by *in vivo* microdialysis was rather similar to that of plasma. The clearance rate was also similar but the peak occurred later.

Following THA (5 mg kg^{-1}) all animals displayed marked central cholinergically mediated behaviour (tremor and chewing) and this is to be expected since the K_i for acetylcholinesterase inhibition by THA is 0.06 μM (Hunter *et al.*, 1989) which is considerably less than both the peak tissue concentration (around 60 μM) and also the extracellular concentration (1.5 μM). However, following this dose of THA no evidence was obtained for a release of dopamine from the striatum. In contrast, amphetamine (5 mg kg^{-1}) induced a marked release of dopamine which was similar in THA-treated and control rats. Our *in vitro* data had indicated that amphetamine and THA were approximately equipotent in releasing dopamine from slices (Robinson *et al.*, 1989). The current results therefore suggest both that the dialysis technique is adequate for detecting release and also that THA, unlike amphetamine, does not induce dopamine release *in vivo* at pharmacologically relevant doses. The addition of 100 mM KCl to the dialysis medium also induced a substantial dopamine release which returned to basal levels on removal of the high KCl. This 'potassium-induced' release of dopamine was also unaffected by THA administration, consistent with previous observations *in vitro* on brain slices (Robinson *et al.*, 1989). Overall the data suggest that the dopamine-releasing action of THA observed *in vitro* does not occur *in vivo*. Nevertheless all these experiments were performed on animals under chloral hydrate anaesthesia and it seemed possible that the anaesthetic might have masked the monoamine releasing actions of THA. It was possible, however, to study 5-HT release from the frontal cortex in unanaesthetized animals. In these experiments, THA was again without effect on the release of 5-HT while a high K^+ concentration evoked a marked enhancement of transmitter release which was unaffected by prior THA administration. Since it had been previously shown that THA was equally effective in stimulating 5-HT and dopamine release *in vitro* (Robinson *et al.*, 1989), it seems unlikely that the failure of THA to enhance dopamine release was due to the anaesthesia.

A further indication that THA does not alter monoamine function *in vivo* was the observation that a high dose of THA (15 mg kg^{-1}) did not affect the concentration of dopamine or its metabolites DOPAC and HVA in the striatum or 5-HT or its metabolite 5-HIAA in the frontal cortex (Robinson *et al.*, 1989). Taken together the present results suggest strongly that THA does not affect monoamine release in the brain at doses which inhibit acetylcholinesterase and which are relevant therapeutically.

The lack of effect on monoamine release is probably explained by examination of the concentration of THA in the brain. The intracellular or tissue concentration seen in the current study if present in the incubation medium would have enhanced monoamine release from brain slices *in vitro*. However, the extracellular concentration *in vivo* was about 30 times lower and would have been ineffective in releasing monoamines from the slices (Robinson *et al.*, 1989). The lack of effect *in vivo* might be explained therefore by the low extracellular concentration that occurs. Similar arguments might also be used to render less likely the suggestions based on *in vitro* studies that *in vivo* THA might interact with the phencyclidine binding site (Albin *et al.*, 1988) and alter γ-aminobutyric acid (GABA) release (de Belleroche & Gardiner, 1986). Indeed one *in vivo* study has already failed to detect altered GABA release following THA administration to rats (Palmer *et al.*, 1990).

It has been proposed that the efficacy of THA in the treatment of demented patients might be due not only to cholinesterase inhibition but also to some other pharmacological effect. The present results make it unlikely that one of these pharmacological effects is enhanced monoamine release. It therefore remains possible that the efficacy of the drug in dementia results solely from its cholinomimetic action coupled with a good pharmacokinetic profile.

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Desensitization and functional antagonism by β-adrenoceptor and muscarinic receptor agonists, respectively: a comparison with receptor alkylation for calculation of apparent agonist affinity

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1 Apparent affinity constants (K_D) for prenalterol, an agonist of low intrinsic efficacy at β_1-adrenoceptors in rat left atria, have been determined by use of receptor desensitization and functional antagonism induced by isoprenaline and carbachol, respectively. The values obtained have been compared to those values estimated with the irreversible β-adrenoceptor antagonist, bromoacetylalprenololmenthane (BAAM).

2 The $-\log K_D$ values for prenalterol estimated by desensitization or irreversible antagonism ranged from 6.8–7.1 and 6.2–7.1, respectively.

3 Carbachol produced functional antagonism of the response to prenalterol even though it was removed before addition of prenalterol. This effect was mediated by M_2-muscarinic receptors. Pretreatment of animals with pertussis toxin did not affect the functional antagonism elicited by carbachol. The apparent K_D value obtained after pre-exposure to carbachol (6.8) was similar to those estimated by use of either alkylation with BAAM or desensitization with isoprenaline (see above).

4 It is concluded that acute desensitization or functional antagonism of responses to agonists of low intrinsic efficacy provides a means to estimate apparent K_D constants. This approach could be useful to characterize receptors for which an irreversible antagonist may not be available.

Keywords: β-adrenoceptors; muscarinic receptors; prenalterol; agonist dissociation constants; BAAM (bromoacetylalprenololmenthane); functional antagonism; desensitization

Introduction

Traditionally, receptors are classified on the basis of differential antagonist affinities. However, concern has been expressed as to the correct manner to classify receptor subtypes (see Black, 1987; Green, 1990 for reviews) since the receptor may exhibit a more stringent structure-activity requirement for the endogenous ligand than for a synthetic ligand, such as antagonists (Kenakin, 1987). The multiplicity of 'pharmacological' sites may reflect the binding of antagonists to accessory membrane sites as well as to the 'receptive' site (Leff & Martin, 1988; Bevan *et al.*, 1989). The unambiguous determination of agonist apparent affinities ($-\log K_D$), therefore, would be useful in receptor characterization. The measurement of affinities for full agonists (Furchgott, 1966), however, requires irreversible antagonists for the receptor in question, which are not available for most receptors or receptor types (Kenakin, 1987). Furthermore, the potential of irreversible antagonists to bind to extra receptor sites in the membrane may influence apparent agonist affinity and consequently selectivity.

An alternative approach to the use of irreversible antagonists for the determination of apparent K_D values for agonists may be to use desensitization. In the sustained presence of high agonist concentrations, many receptor types undergo desensitization. The effects are qualitatively similar to those of receptor alkylation, i.e., the concentration-response curves are dextrally shifted and the maxima, upon depletion of the spare receptors, are depressed (Eglen *et al.*, 1987; Herepath & Broadley, 1990). However, unless the desensitization process is homologous, i.e. tissue responses to a single receptor type are desensitized, then null methods, which assume equal responses with equal degrees of receptor occupancy (Furchgott, 1966), may not apply since the receptor coupling system, in addition to the active receptor population, could be affected (Kenakin, 1987; Eglen *et al.*, 1987).

Application of such null methods to heterologous desensitization of the ileal M_3-muscarinic receptor results in apparent K_D values for several full agonists but not partial agonists, that were incorrect in comparison to the apparent K_D values estimated by alkylation (Eglen *et al.*, 1987). In cultured AH31 cells, the effect of homologous β_2-adrenoceptor desensitization was, however, analogous to that seen after receptor alkylation with N^8(bromoacetyl)-N-[3-(4-indolyoxy)-2-hydroxy-propyl]-(2)-1,8-diaminomenthane (BIM; Lohse, 1990). Moreover, apparent agonist K_D values estimated by the two methods were in good agreement.

The use of homologous densitization has been examined in the present study. Positive inotropic responses to β-adrenoceptor agonists in rat isolated left atria were used as a model to test this approach for several reasons. Firstly, rat atria have been well characterized in terms of the receptor populations by both radioligand and functional binding techniques (Juberg *et al.*, 1985; Montgomery *et al.*, 1988a; Herepath & Broadley, 1990). Secondly, short term β-adrenoceptor desensitization in cardiac tissue is generally homologous (see Harden, 1983 for review). Thirdly, an irreversible β-adrenoceptor antagonist, bromoacetylalprenololmenthane (BAAM), was available with which to compare directly apparent K_D values calculated by desensitization. Prenalterol, an agonist of low relative efficacy, was employed because (a) its affinity at β-adrenoceptors has been measured by both functional and radioligand binding methods (Kenakin & Beek, 1982; 1984; Kenakin, 1987; Gurden *et al.*, 1989), and (b) errors involved in the estimation of the apparent K_D by the method of Furchgott (1966) for partial agonists are less than those associated with full agonists (Eglen *et al.*, 1987; Leff *et al.*, 1990a,b).

A second aspect of this paper concerns functional antagonism. Stimulation of muscarinic receptors has been shown to produce functional antagonism of β-adrenoceptor responses (O'Donnell & Wanstall, 1977; Broadley & Nicholson, 1979; Buckner & Saini, 1975; Leff *et al.*, 1985; Molenaar & Malta,

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1986), also resulting in effects qualitatively similar to those seen after receptor alkylation described above. There are, however, theoretical limitations to the use of functional antagonism to estimate apparent K_D values, particularly in cardiac tissue (MacKay, 1981; Leff et al., 1985; Molenaar & Malta, 1986). Notably, the baseline tension developed by isolated atrial tissue in the presence of carbachol is reduced, thereby changing the state of the tissue and invalidating one of the assumptions of the null hypothesis developed by Furchgott (1966). In the present study, a procedure modified from that described by Broadley & Nicholson (1979) has been used, i.e., carbachol was removed from the organic bath prior to construction of the second concentration-response curve, allowing the baseline tension to re-attain control values. The apparent K_D values estimated with these data have been compared with those estimated by use of desensitization and receptor alkylation.

Some of these data have been presented in a preliminary form to the British Pharmacological Society (Montgomery et al., 1988a) and to the American Society for Pharmacology and Experimental Therapeutics (Montgomery et al., 1988b).

Methods

Tissue preparation

Rats (male, Sprague-Dawley, 200–250 g) were pretreated with reserpine (2.5 mg kg^{-1}, i.p., twice daily, for two days; the last dose was administered 18 h before tissue removal). Preliminary experiments showed that 0.1 mM tyramine did not elicit a positive inotropic response in isolated atria from reserpine-treated animals, suggesting a substantial reduction in endogenous catecholamine concentrations. Rats were lightly anaesthetized with CO_2 and decapitated. Isolated atria were then placed in warm (34°C) oxygenated (95% O_2:5% CO_2) Krebs-Henseleit physiological salt solution (composition mM: NaCl 118, KCl 4.7, $CaCl_2 \cdot 6H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.6, $NaHCO_3$ 24.9, KH_2PO_4 1.2, glucose 5.6, EDTA 0.05) and cleaned of adhering connective tissue. The atria were then placed under 0.5 g tension in 10 ml organ baths containing Krebs solution (37°C) and placed between parallel platinum electrodes (Eglen et al., 1988). The atria were electrically paced during the experiments (2 Hz, 5 ms duration, threshold voltage +12%) with electrical stimulation provided by a Grass S88 stimulator via a Buxco signal splitter. Tissues were allowed 60 min to equilibrate with overflow washing at 10 min intervals.

Receptor characterization

To characterize the β-adrenoceptor population present in the rat left atria, affinities i.e., $-\log K_B$ values, for the antagonists propranolol (nonselective), atenolol (β_1-selective), and ICI 118,551 (β_2-selective) were determined. Concentration-response curves to isoprenaline were established in the presence and absence of these antagonists after 60 min of equilibration (with regular washing at 15 min intervals). The muscarinic receptor subtype mediating negative inotropic responses to carbachol was also characterized (see Eglen & Whiting, 1986, for review) by determining $-\log K_B$ values for atropine (nonselective), pirenzepine (M_1-muscarinic receptor-selective), methoctramine (M_2-muscarinic receptor-selective), and para-fluoro-hexahydrosiladiphenidol (*p*-F-HHSiD; M_3-muscarinic receptor-selective).

Receptor inactivation studies

After the equilibration period, a single dose of 100 nM isoprenaline was added for 5 min to obtain an estimate of the maximum response of the tissue. The tissues were then washed at 10 min intervals over the following 60 min and allowed to re-attain baseline tension. A cumulative concentration-response curve to prenalterol was then established in the cumulative fashion (Van Rossum, 1963) with incremental increases of concentration spaced at 0.5 log intervals. After the maximum response was attained the tissues were washed by overflow at 10 min intervals over the succeeding 30 min and allowed to re-attain baseline tension.

The irreversible β-adrenoceptor antagonist BAAM (Posner et al., 1984) was added to the organ baths for a period of 60 min (32–3200 nM) with washing at 10 min intervals. Each preparation was exposed to only one concentration of BAAM. The tissues were washed at 10 min intervals over the succeeding 30 min and a second cumulative concentration-response curve to prenalterol was then established. The apparent K_D value was then estimated by the method of Furchgott (1966) as described below.

The apparent K_D value was also estimated by the method of Furchgott & Bursztyn (1967). This latter method reduces the efficacy of a partial agonist thereby approximating it to a competitive antagonist. In separate experiments to those described above, tissues were initially exposed to either 32 nM or 3.2 μM BAAM. After a 30 min washout period, concentration-response curves to isoprenaline were then constructed in the presence or absence of 10 μM prenalterol (with a 60 min equilibration period). The dextral shift in the concentration-response curves to isoprenaline was then used to calculate the apparent K_D value for prenalterol (see below). It should be noted that the constants calculated are only apparent, since drug and receptor $\rightleftharpoons$ drug/receptor complex is not a realistic description of agonist action at G-protein-coupled receptors.

Receptor desensitization studies

The procedure employed was similar to that described above except that isoprenaline (3.2–560 nM) was substituted for BAAM, and left in contact with the preparations for 120 min. This period was chosen since Herepath & Broadley (1990) have shown that exposure greater than 4 h resulted in irreversible desensitization (i.e. down regulation). After 120 min exposure, washing at 10 min intervals was continued for the next 60 min or until baseline tension was restored. A second concentration-response curve to prenalterol was then established. In separate experiments, apparent K_D values were also estimated by the method of Furchgott & Bursztyn (1967). The tissues were initially exposed to isoprenaline (3200 nM), and, after washout, a concentration-response curve to isoprenaline was constructed in the presence of 10 μM prenalterol.

Functional antagonism

The procedure followed was similar to that described above for isoprenaline, except that the tissues were exposed to carbachol (0.1 μM–0.1 mM) for 120 min. After exposure, the tissues were washed at 10 min intervals over the next 60 min. In all experiments, the baseline tension was returned to pre-carbachol values prior to the construction of a second curve to prenalterol. In a separate series of experiments, the muscarinic receptor subtype was characterized. The preparations were exposed simultaneously to atropine (320 nM) and carbachol (10 μM) for 120 min, prior to the construction of the second concentration-response curve to prenalterol. Similar experiments were performed with pirenzepine at a concentration (100 nM) that has been shown to antagonize selectively M_1-muscarinic receptors (Eglen & Whiting, 1986).

Pretreatment with pertussis toxin

Pertussis toxin (10 μg kg^{-1}) or vehicle was injected via the tail vein 48 h prior to the removal of tissues (Eglen et al., 1988). This regimen was shown to abolish the negative inotropic responses to muscarinic agonists due to ADP-ribrosylation of the G_i protein (Tajima et al., 1987; Eglen et al., 1988).

Effect on responses to forskolin

To estimate the specificity of the inactivating effects of BAAM, isoprenaline-induced desensitization, or carbachol-induced functional antagonism, concentration-response curves to the adenylate cyclase activator, forskolin (1 nM–10 μM, 5 min exposure at each concentration) were established before and after exposure to each of these agents. The method and conditions were similar to those described above.

Measurement of responses

All tissue responses were determined as changes in isometric tension (mg). Responses were measured with a Grass FT03 isometric force transducer and displayed on a Grass 8 channel recorder. These values were determined at each agonist concentration. If necessary, values on the post-exposure curves were corrected for any time-dependent changes (2–5 fold) in sensitivity derived from corresponding control studies. The increases in developed tension were then calculated as a percentage of the response to 100 nM isoprenaline.

Analysis of responses

The potencies and maximal responses to prenalterol from the pre- and post-exposure curves were defined by the EC_{50} and percentage of the maximum of the pre-exposure curve. This latter parameter was derived by non-linear iterative curve fitting procedures (Parker & Waud, 1971) using RS1 software (BBN Corporation, MA, U.S.A.). Antagonist equilibrium dissociation constants ($-\log K_B$) were calculated by the method of Gaddum (1937):

$$K_B = \frac{[\text{antagonist concentration}]}{(\text{concentration-ratio} - 1)}$$

Apparent equilibrium dissociation constants (K_D) for prenalterol were calculated in two ways. All pre- and post-exposure curves to BAAM, isoprenaline or carbachol, were employed to calculate apparent K_D values by the method of Furchgott (1966):

$$\frac{1}{[A]} = \frac{1}{[A^1]}\frac{1}{q} + \frac{1}{K_D}\frac{(1-q)}{q}$$

where q is the fraction of receptors remaining after desensitization, K_D is the apparent equilibrium dissociation constant, and [A] and [A^1] are equally effective concentrations of prenalterol before and after exposure. These equieffective concentrations were determined at 10% increments of response. These values were then plotted in a double reciprocal fashion, and the apparent K_D value was derived after exposure. These equieffective concentrations were determined at 10% increments of response and the values were then plotted in a double reciprocal fashion, and the apparent K_D value derived from the resulting straight line as (slope − 1)/intercept.

In separate experiments following receptor inactivation or desensitization, values were derived by comparing curves constructed to isoprenaline in the presence or absence of prenalterol (10–100 μM); the latter acting as an antagonist. In this instance, the following relationship was employed

$$[A] = \frac{[A^1]}{1 + ([P]/K_D)}$$

where [A] and [A^1] are concentrations of the full agonist (isoprenaline) in the absence and presence of the partial agonist P (prenalterol). The apparent equilibrium dissociation constant for prenalterol (K_D) was derived from the relationship [P] × slope/(1 − slope). This relationship is a modification of the equation of Gaddum (1937) described above.

Statistical analysis

All values quoted are means ± s.e.mean, $n = 4$–6. Statistical differences were assessed by use of the Student's unpaired t test, with $P < 0.05$ considered to be significant.

Drugs used

The following compounds were employed: atenolol, ICI 118,551 (erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol; ICI plc, Alderley Edge, U.K.), BAAM, forskolin, methoctramine (Research Biochemicals Inc., Natick, MA, U.S.A.), pertussis toxin (Peninsula Peptides, San Diego, CA, U.S.A.), pirenzepine (Boots plc, Nottingham, U.K.), and prenalterol (A.B. Hassle, Molndal, Sweden). Racemic *p*-F-HHSiD (*para*-fluoro-hexahydro-siladiphenidol) was synthesized by the Institute of Organic Chemistry, Syntex, Palo Alto, CA, U.S.A. The remaining compounds were obtained from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A.).

Results

Isoprenaline and prenalterol elicited concentration-dependent positive inotropic responses. The maximal responses to prenalterol (relative to the maximal response of isoprenaline) varied between preparations, ranging from 55 to 112%. As a result, initial concentration-response curves to prenalterol were always established in each preparation prior to exposure of the tissues to BAAM, isoprenaline, or carbachol, thereby providing an internal control curve for each preparation.

β-Adrenoceptor and muscarinic receptor characterization

Antagonist affinities ($-\log K_B$) were derived to characterize the populations of β- and muscarinic receptors present (Table 1). Propranolol and atenolol (100 nM) shifted the concentration-response curve to isoprenaline to the right in a parallel fashion, whereas no effect was observed with ICI 118,551 (100 nM). Concentration-response curves to carbachol were shifted to the right in a parallel fashion by atropine (100 nM), methoctramine (100 nM) and pirenzepine (3000 nM) but not by *p*-F-HHSiD (100 nM). In all of these studies, no diminution in the maximal response to either isoprenaline or carbachol was observed.

β-Adrenoceptor inactivation

BAAM, (32 and 100 nM) reduced both the potency and maxima of the concentration-response curve to prenalterol (Figure 1, Table 2). After exposure to 320 and 1000 nM BAAM, no further significant decreases in maxima were observed. Exposure to 3200 nM, however, resulted in a further decrease in maxima, which was accompanied by a larger dextral shift. The apparent affinities ($-\log K_D$) for prenalterol were similar

Table 1 Apparent antagonist affinities ($-\log K_B$) in rat left atria

Response	*Agonist*	*Antagonist*	$-log\ K_B$
Positive inotropic	Isoprenaline	Propranolol	8.6 ± 0.1
	Isoprenaline	Atenolol	8.0 ± 0.1
	Isoprenaline	ICI 118,551	<7.0
Negative inotropic	Carbachol	Atropine	8.6 ± 0.1
	Carbachol	Methoctramine	7.9 ± 0.1
	Carbachol	*p*-F-HHSiD	<7.0
	Carbachol	Pirenzepine	6.9 ± 0.1

Values are mean ± s.e.mean, $n = 6$. All antagonists were studied at a concentration of 0.1 μM with the exception of pirenzepine (0.3 μM). *p*-F-HHSiD-*para*fluorohexahydrosiladiphenidol.

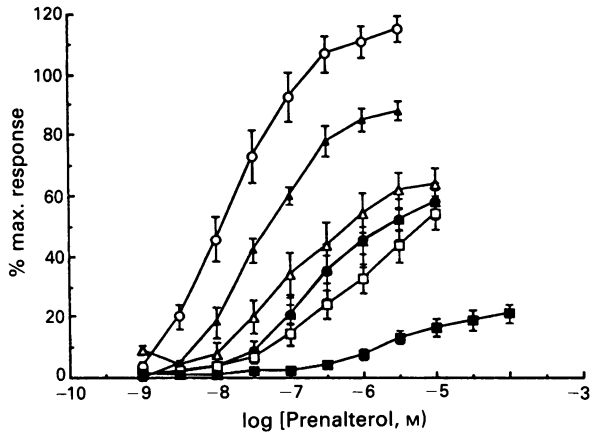

Figure 1 Responses to prenalterol in rat left atria in the absence and after exposure to bromoacetylalprenolol menthane (BAAM). The concentrations of BAAM were 32 nM (▲), 0.1 μM (△), 0.32 μM (●), 1 μM (□) and 3.2 μM (■). Points show mean values with vertical bars indicating s.e.mean.

after inactivation by 32 and 100 nM BAAM, but these values progressively decreased after exposure to higher concentrations (Table 2).

The apparent K_D values were also estimated by the method of Furchgott & Bursztyn (1967) following pretreatment of the tissues with BAAM (32 and 3200 nM). The apparent $-\log K_D$ values were significantly ($P < 0.05$) different (7.07 ± 0.05 and 6.02 ± 0.08, respectively) in tissues exposed to 32 or 3200 nM BAAM. These apparent $-\log K_D$ values were similar, however, to those determined by the method of Furchgott (1966) using these concentrations of BAAM (6.7 and 6.2, respectively; Table 2).

Isoprenaline-induced desensitization

After exposure to isoprenaline (3.2–560 nM) for 120 min, there was a concentration-dependent diminution of the maxima of the concentration-response curve to prenalterol (Table 3). The apparent affinities ($-\log K_D$), in contrast to those estimated with BAAM, were similar after exposure to each concentration of isoprenaline. The apparent K_D value estimated by the method of Furchgott & Bursztyn (1967) following exposure of the tissues to 560 nM isoprenaline was 7.14 ± 0.03.

Functional antagonism

Exposure of the tissues to 0.32 and 1 μM carbachol, followed by a 30 min washout period, did not significantly inhibit the responses to prenalterol (Table 4). However, after exposure to 10 μM carbachol and a similar washout period, the potency and the maximum of the prenalterol concentration-response curve were significantly reduced (Table 4). The affinity estimated after exposure to 10 μM carbachol was similar to that obtained with both BAAM (32–100 nM) and isoprenaline-induced desensitization (Table 4).

In some experiments, tissues were exposed to both atropine (320 nM) and carbachol (10 μM) for 3 h, prior to construction of the second concentration-response curve to prenalterol. Under these conditions, the desensitizing effects of carbachol were reversed (Table 5). Pirenzepine (100 nM), in contrast, did not reverse the carbachol-induced desensitization of responses to prenalterol (Table 5). Pretreatment of the animals with pertussis toxin (10 mg kg^{-1}, i.v.) abolished negative inotropic responses to carbachol, but revealed a positive inotropic response at carbachol concentrations $> 10^{-5}$ M (Figure 2). Moreover, pertussis toxin pretreatment did not alter the functional antagonism by carbachol of the responses to prenalterol (Table 5, Figure 3). The positive inotropic responses to prenalterol or isoprenaline alone were unaffected by pertussis toxin pretreatment (data not shown).

In order to obtain some measure of the specificity of the effects of BAAM or isoprenaline on functional antagonism, concentration-response curves to forskolin were constructed following pretreatment with the agents. It was observed that pre-exposure to BAAM (3200 nM; 60 min), isoprenaline (560 nM; 120 min), or carbachol (10 μM; 180 min) did significantly ($P < 0.05$), but not meaningfully, affect either the potency or maximal responses observed with forskolin (Table 6).

Table 2 Potencies ($-\log EC_{50}$), maximal responses, and apparent affinities ($-\log K_D$) of prenalterol after receptor inactivation by bromoacetylalprenolol menthane (BAAM)

BAAM concentration (nM)	*−log EC_{50}*	*% max. isoprenaline response*	*Max. isoprenaline response* (mg)	*−log K_D*	*q*
Control	7.8 ± 0.1	112 ± 8	670 ± 40	—	—
32	7.4 ± 0.1	84 ± 5	693 ± 50	6.7 ± 0.1	0.37
100	6.9 ± 0.1	62 ± 8	730 ± 90	7.0 ± 0.1	0.15
320	6.5 ± 0.1	57 ± 5	644 ± 60	6.5 ± 0.1	0.05
1000	6.3 ± 0.1	59 ± 6	679 ± 73	6.2 ± 0.1	0.03
3200	5.6 ± 0.1	20 ± 3	632 ± 45	6.2 ± 0.1	0.004

Values are mean ± s.e.mean, $n = 4$–8. Control values are those determined prior to exposure to BAAM. Equilibrium dissociation constants (K_D) and proportion of receptors remaining after inactivation (q) were derived after the method of Furchgott (1966).

Table 3 Potencies ($-\log EC_{50}$), maximal responses, and apparent affinities ($-\log K_D$) of prenalterol after densensitization by isoprenaline

Isoprenaline concentration (nM)	*−log EC_{50}*	*% Max isoprenaline response*	*Max isoprenaline response* (mg)	*−log K_D*	*q*
Control	7.6 ± 0.1	80 ± 5	749 ± 68	—	—
3.2	7.0 ± 0.1	52 ± 8	639 ± 94	7.1 ± 0.1	0.52
32	6.8 ± 0.1	36 ± 5	791 ± 83	6.9 ± 0.1	0.34
320	6.7 ± 0.1	30 ± 7	753 ± 53	6.9 ± 0.1	0.15
560	6.5 ± 0.1	21 ± 4	721 ± 46	6.8 ± 0.1	0.08

Values are mean ± s.e.mean, $n = 4$–8. Control values are those determined prior to exposure to isoprenaline. See legend to Table 2 for explanation of K_D and q values. Apparent $-\log K_D$ values estimated by method of Furchgott (1966).

Table 4 Potencies ($-\log EC_{50}$) and apparent affinities ($-\log K_D$) of prenalterol maximal responses after functional antagonism by carbachol

Carbachol concentration (μM)	*$-\log EC_{50}$*	*% Max isoprenaline response*	*Max isoprenaline response* (mg)	*$-\log K_D$*	*q*
Control	7.20 ± 0.1	58 ± 5	695 ± 35	—	—
0.32	7.16 ± 0.1	52 ± 6	778 ± 67	NC	
1	6.88 ± 0.1	43 ± 4	650 ± 97	NC	
10	6.74 ± 0.1	18 ± 3	800 ± 38	6.8 ± 0.1	0.17

Values are mean ± s.e.mean, $n = 4$–6. NC = not calculated. Control values are those determined prior to exposure to carbachol. Carbachol (10 μM) was applied to the atria for 120 min and then washed out over a period of 60 min. See legend of Table 2 for explanation of K_D and q values.

Table 5 Effect of pre-exposure to carbachol after various conditions on responses to prenalterol

Pre-exposure conditions	*$-\log EC_{50}$*	*Max % isoprenaline response*	*Max isoprenaline response* (mg)
Pre carbachol	7.20 ± 0.08	58 ± 5	695 ± 35
Post carbachol	6.74 ± 0.08	18 ± 3	800 ± 38
Post carbachol (+0.32 μM atropine)	6.79 ± 0.05	46 ± 5	825 ± 56
Post carbachol (+0.1 μM pirenzepine)	6.95 ± 0.11	19 ± 8	588 ± 80
Post carbachol (+vehicle)[a]	6.93 ± 0.05	23 ± 3	506 ± 99
Post carbachol (+PTx pretreatment)	7.04 ± 0.03	24 ± 8	492 ± 99

Values are mean ± s.e.mean, $n = 4$–6. The maximal isoprenaline concentration employed was 0.1 μM. The carbachol (10 μM) exposure period was 180 min. PTx: pertussis toxin.
[a] Vehicle-treated animals were pretreated with 0.3 ml H_2O injected 3 h prior to tissue removal.

Table 6 Effect of pre-exposure to bromoacetylalprenolol menthane (BAAM), isoprenaline, or carbachol on responses to forskolin

Pre-exposure conditions	*$-\log EC_{50}$*	*Max. % isoprenaline response*	*Max. isoprenaline response* (mg)
Control	6.26 ± 0.09	96 ± 7	748 ± 63
BAAM (3200 nM; 60 min)	6.55 ± 0.13	93 ± 4	734 ± 112
Isoprenaline (560 nM; 120 min)	6.34 ± 0.11	98 ± 7	842 ± 98
Carbachol (10,000 nM; 180 min)	5.89 ± 0.04*	104 ± 8	970 ± 49

Values are mean ± s.e.mean, $n = 4$–8. Atria were pretreated as indicated prior to construction of forskolin concentration-response curves.
* Significantly different ($P < 0.05$) from control value.

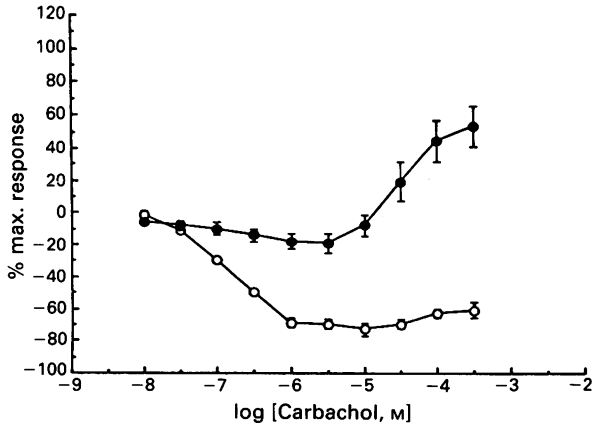

Figure 2 Effects of pretreatment of animals with pertussis toxin (●) or vehicle (○) on inotropic responses to carbachol in rat left atria. Points show mean values with vertical bars indicating s.e.mean.

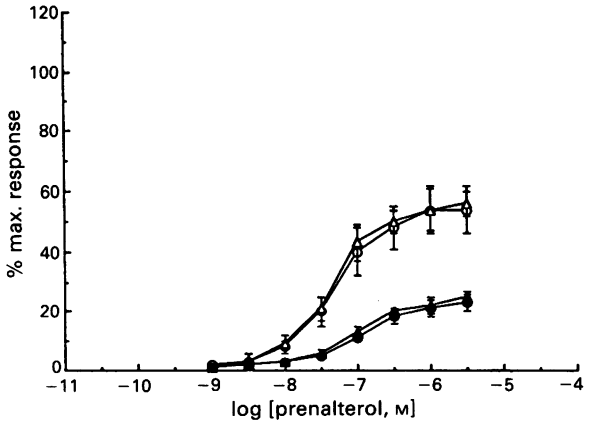

Figure 3 Effects of pre-exposure of rat left atria to carbachol on responses to prenalterol in vehicle-treated (●) or pertussis toxin-treated (▲) animals. Effects in control tissues (no carbachol treatment) are also shown in pertussin toxin-treated (○) or vehicle-treated (△) animals. Points show mean values with vertical bars indicating s.e.mean.

Discussion

Affinity and efficacy are fundamental parameters in the analysis of agonist/receptor interactions and receptor alkylation is extensively employed to measure these parameters. Many receptor systems lack such selective alkylating agents, thus precluding the use of this approach. The classification of the many receptors, therefore, is based on the differential affinities of competitive reversible antagonists. In the present study desensitization has been used as an alternative method to determine agonist affinity since many receptors undergo desensitization and the consequences are similar, at least temporarily, to those observed after alkylation. If, however, desensitization influences post-receptor events, then incorrect estimates of agonist affinities may result (MacKay, 1981; Leff *et al.*, 1985; Eglen *et al.*, 1987; Lohse, 1990).

In order to characterize the adrenoceptor and muscarinic receptor populations present, antagonist studies were undertaken. The affinities ($-\log K_B$) of propranolol (non-selective), atenolol (β_1-adrenoceptor selective), and ICI 118,551 (β_2-adrenoceptor selective) suggest that the positive inotropic response was mediated through β_1-adrenoceptors while the data obtained with atropine (nonselective), pirenzepine (M_1-muscarinic receptor-selective), methoctramine (M_2-muscarinic receptor-selective), and *p*-F-HHSiD (M_3-muscarinic receptor-selective) suggest that the negative inotropic responses to carbachol were mediated through an M_2-muscarinic receptor (see also Juberg *et al.*, 1985; Eglen & Whiting, 1986, for pA_2 values and further references).

The effects of BAAM on the responses to prenalterol were different from the effects of isoprenaline-induced desensitization. The apparent K_D values were independent of the level of inactivation only for isoprenaline-induced desensitization but not for alkylation with BAAM. The apparent K_D values (6.8–7.1) measured by the methods of Furchgott (1966) or Furchgott & Bursztyn (1967) agreed with the apparent affinity ($pA_2 = 6.8$) reported by Kenakin & Beek (1984), who used prenalterol as a competitive antagonist. These values were also similar to those obtained in the rat atria with radioligand binding techniques ($-\log K_1 = 6.81$, Juberg *et al.*, 1985). In guinea-pig left atria, prenalterol acted as a competitive antagonist with a pA_2 value of 7.2 (Molenaar *et al.*, 1985). Taken together, these data suggest that reproducible apparent K_D values for prenalterol can be estimated by the methods described by Furchgott (1966) or Furchgott & Bursztyn (1967). The theoretical limitations recently discussed in the literature (see Mackay, 1988; 1990; Kenakin, 1989 for reviews) may not apply. Similar findings have been reported by Leff *et al.* (1990a,b) and Eglen *et al.* (1987) for the affinity of pilocarpine at the cardiac M_2 or ileal M_3 muscarinic receptor, respectively.

BAAM at higher concentrations proved an unreliable tool, since the apparent K_D values varied. BAAM (10–30 μM) has been shown to act as a competitive antagonist of responses in the guinea-pig atria to isoprenaline and the partial agonist oxymethylene-isoprenaline (Ng & Malta, 1989). Black *et al.* (1985) found that, at concentrations greater than 10 μM, BAAM depressed the basal rate of the beating of rat atria. These observations, together with the data from the present study, discourage the use of BAAM at concentrations greater than 320 nM. This is in contrast to data reported by Doggrell (1989), who has employed 3 μM BAAM to estimate an apparent K_D value for isoprenaline at β_1-adrenoceptors in the right ventricle of the rat.

Acute desensitization by isoprenaline also permitted a reliable estimation of the apparent K_D value for prenalterol (this study). The apparent K_D values obtained by this method were similar to those reported in the literature (see above) and to those estimated with low concentrations of BAAM. Exposure to isoprenaline for 180 min did not affect either the potency or the maximal response to forskolin. This indicated that the isoprenaline-induced β-adrenoceptor desensitization did not directly affect the catalytic subunit of adenylate cyclase, though it should be noted that this does not exclude the possibility of changes in the G_S guanine nucleotide binding protein.

In the experiments on functional antagonism by carbachol, the procedure described by Broadley & Nicholson (1979) was modified by removing carbachol from the tissue by extensive washing before exposure to prenalterol. The inotropic responses to prenalterol were still inhibited, and analysis yielded an apparent K_D value similar to that reported in the literature and that from BAAM inactivation or isoprenaline-induced desensitization (this study). There was a significant but not meaningful inhibition of forskolin activity after carbachol exposure, implying that the functional antagonism was unrelated to effects on the catalytic unit of adenylate cyclase. These data suggest that under the correct experimental conditions, functional antagonism may accurately measure agonist affinity, at least for partial agonists such as prenalterol.

Other workers using carbachol in atria (Broadley & Nicholson, 1979) or trachea (Buckner & Saini, 1975; O'Donnell & Wanstall, 1977) have utilized carbachol, present throughout the experiment, to produce functional antagonism responses to β-adrenoceptor agonists. However, the apparent K_D values for isoprenaline estimated in this fashion differed from those estimated in radioligand binding studies (see Leff *et al.*, 1985 for references). Molenaar & Malta (1986) have argued that these disparities undermine the validity of functional antagonism for determination of β-adrenoceptor agonist affinities. Kenakin (1987), in contrast, has shown that the latter method may be applicable when the efficacy of a partial agonist is depressed to such an extent that it acts as a competitive antagonist.

Pretreatment of animals with pertussis toxin, at a dose which has been shown (Eglen *et al.*, 1988) to abolish the negative inotropic response to carbachol, failed to influence the carbachol-induced desensitization. This suggested that the M_2-muscarinic receptor responsible for carbachol-induced β_1-adrenoceptor desensitization was not coupled via the guanine nucleotide binding protein, G_i. Moreover, sustained inhibition of adenylate cyclase was not a prerequisite for the desensitization, since this was observed even after removal of the carbachol (where baseline developed tension had return to control levels). The insensitivity to pertussis toxin of both functional antagonism by carbachol (this study) and the inotropic response to muscarinic agonists (Eglen *et al.*, 1988), suggests a common mechanism. Furthermore, the concentration range over which carbachol elicits these effects is also similar (desensitization: 1–100 μM; positive inotropy: 1–1000 μM). Taken together, these data suggest that the cardiac M_2-muscarinic receptor elicits multiple responses (negative, positive inotropy, β-adrenoceptor densensitization) dependent upon the G proteins to which it is coupled. This provides further evidence for promiscuity of G protein coupling by the M_2-muscarinic receptor, as recently suggested by Kenakin & Boselli (1990).

In conclusion, the present study has demonstrated that desensitization with β_1-adrenoceptor agonists or functional antagonism by M_2-muscarinic receptor agonists can be used to estimate reproducible, apparent K_D values for prenalterol. The use of these methods to derive apparent K_D values may have a more general utility, specifically in instances where the agonist exhibits low intrinsic efficacy.

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Modulation of gastric contractions in response to tachykinins and bethanechol by extrinsic nerves

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1 Extrinsic reflexes elicited by changes in gastric wall tension play an important role in regulating gastric tone. The present study investigated whether such reflexes modulate gastric contractions induced by close arterially administered neurokinin A (NKA), substance P (SP), SP-methylester and bethanechol in anaesthetized rats.

2 Reflex pathways were acutely interrupted by either subdiaphragmatic vagotomy or prevertebral ganglionectomy. C-fibre afferent nerve activity was abolished by pretreating rats with capsaicin 10 to 16 days before the experiments.

3 The order of potency in inducing gastric contractions was NKA > SP > bethanechol. SP-methylester was markedly less effective than SP and its effects did not fit sigmoid dose-response curves (DRCs). The maximal responses to NKA, SP, and bethanechol were similar, whilst the DRC for SP was significantly flatter than those for NKA or bethanechol. Pretreatment of the rats with the peptidase inhibitors phosphoramidon or captopril did not increase the contractile response to SP.

4 Prevertebral ganglionectomy had no significant effect on the DRCs for SP and NKA, whereas vagotomy shifted the DRCs for all three test substances to the left.

5 Capsaicin pretreatment did not change the DRC for NKA in rats with intact vagus but shifted that for bethanechol to the left. The leftward shift of the DRC for NKA caused by vagotomy was prevented in capsaicin-pretreated rats whereas the vagotomy-induced shift of the DRC for bethanechol remained unaltered. The shift of the DRC for SP seen in response to vagotomy was only slightly reduced by capsaicin pretreatment.

6 These data may be interpreted as demonstrating two neuronal mechanisms for modulating drug-induced gastric contractions. First, the contractions themselves activate a vago-vagal negative feedback involving capsaicin-sensitive afferents. Second, NKA, and to a lesser degree SP, seem to induce a non-vagal non-splanchnic mechanism which via capsaicin-sensitive afferent neurones reinforces tachykinin-induced gastric contractions.

Keywords: substance P; neurokinin A; bethanechol; gastric motility; afferent; vagus

Introduction

Reflex nerve activity plays a major role in regulating gastric tone. Probably the most important of these reflexes is the so-called adaptive relaxation, i.e. gastric relaxation initiated by gastric distension, involving afferent and efferent branches in the vagus nerves (Abrahamsson & Jansson, 1973). Relaxant responses are also evoked by selective distension of the antrum, one component being a vago-vagal reflex, the other a spinal one (Abrahamsson, 1973a,b). Such a reflex was also elicited by close arterial injection of acetylcholine to the antrum (Abrahamsson, 1973b). Antral contractions in response to distension of the antrum or corpus have also been described (Andrews *et al.*, 1980; Grundy *et al.*, 1986).

In view of the importance of such extrinsic reflexes, the possibility was envisaged that gastric contractions brought about by exogenously administered motor stimulants might be modulated by similar mechanisms. The tachykinins neurokinin A (NKA) and substance P (SP) were chosen as test substances since they have been shown to be involved in the regulation of gastrointestinal motility (see Barthó & Holzer, 1985; Holzer-Petsche *et al.*, 1987; Koelbel *et al.*, 1988; Allescher *et al.*, 1989). NKA contracts the rat stomach through receptors on the smooth muscle, whereas SP acts in part via cholinergic neurones (Holzer-Petsche *et al.*, 1987). In order to gain information as to the type of tachykinin receptor involved, the NK_1 receptor agonist, SP-methylester (Watson *et al.*, 1983) was administered. Furthermore, bethanechol, a muscarinic agonist mimicking the action of acetylcholine on smooth muscle (see Holzer-Petsche *et al.*, 1987) was also tested. Extrinsic modulation of the effects of these substances could be effected in two ways: either by a tonic influence from the autonomic nervous system or by nervous reflexes involving afferent and efferent neurones. Since gastric afferents mediating various reflexes have been shown to be sensitive to the sensory neurotoxin capsaicin (McCann *et al.*, 1988; Raybould & Taché, 1988; 1989), this latter possibility was investigated by the use of the neurotoxic action of capsaicin on sensory neurones.

A possible reflex regulation might not only be elicited by the motor effect of, but also by direct chemical stimulation of afferent nerve endings by, the tachykinins (see Lew & Longhurst, 1986; Maggi *et al.*, 1986; Cervero & Sharkey, 1988). To differentiate between these possibilities, experiments were performed with bethanechol, since muscarinic agonists have been shown not to stimulate visceral or cutaneous afferent nerve endings (Armett & Ritchie, 1961; Juan, 1982). Metabolic mechanisms in the blood might also influence the drug on its way to the target organ. SP has been shown to be eliminated from rat blood much faster than NKA (Holzer-Petsche *et al.*, 1988). To test whether the rapid breakdown of SP might account for the lower potency of SP compared with NKA (Holzer-Petsche *et al.*, 1987), SP was tested in rats pretreated with the peptidase inhibitors phosphoramidon or captopril (see Bunnett, 1987).

Methods

Animals

Sprague-Dawley rats (Institut für Versuchstierkunde, Himberg, Austria) of either sex weighing 200–250 g were used. Before the experiments the rats were fasted overnight but had free access to water.

Experimental procedure

The rats were anaesthetized with urethane (1.25 g kg^{-1}, i.p.) and the trachea was cannulated to keep a patent airway. Blood pressure was monitored from a carotid artery. The oesophagus was ligated in the neck. In the experiments with ganglionectomized rats, the abdominal aorta was cannulated so that the tip of the catheter lay at the branching of the coeliac artery. Through this cannula, 154 mM NaCl was continuously infused to the stomach at a rate of 60 μl min^{-1}. For drug administration the infusion was changed from saline to the appropriate drug solution for 3 min. Sequential dose-response curves for SP and NKA were constructed in the same rat with intervals of at least 20 min between consecutive additions. In the experiments with vagotomized or capsaicin-pretreated rats, a retrograde catheter was inserted into the splenic artery for close arterial drug injection as a bolus of 100 μl. In this instance, only one substance was tested in each rat, with intervals of at least 20 min between increasing doses.

Gastric motility was measured as intragastric pressure by means of a pressure transducer attached to a cannula placed into the stomach through an incision in the duodenum and fixed by a ligation around the pylorus. The signals were recorded on a pen recorder. Fifteen minutes after the end of the operation, the stomach was rapidly filled with 3 ml saline and drug administration was begun 20 min later.

In order to eliminate possible reflex loops, two types of lesions were performed immediately before the experiments: for subdiaphragmatic vagotomy, all nerve fibres along the distal oesophagus were severed under the microscope. The reflex pathway via the splanchnic nerves was interrupted by blunt removal of the coeliac-superior mesenteric ganglion complex ('ganglionectomy'). Control animals were sham-operated.

Pretreatment with peptidase inhibitors

In order to inhibit peptidases possibly involved in the breakdown of SP, captopril was administered i.v. at a dose of 10 μmol kg^{-1} 2 min (Shore *et al.*, 1988) before SP or, as after 2 min no effect on blood pressure was observed, 30 min before SP. Phosphoramidon was administered i.v. at 2 or 20 μmol kg^{-1} 2 min before SP (Thompson & Sheppard, 1988). Since i.v. administration of the inhibitors was ineffective, both inhibitors were also administered close arterially into the splenic artery (0.5 μmol captopril or 1 μmol phosphoramidon) 30 s before the i.a. test dose of SP, in order to achieve the same site and time of action of the peptidase inhibitors as of SP.

Capsaicin pretreatment

In order to eliminate the function of small diameter afferent nerve fibres, adult rats were treated s.c. with 125 mg kg^{-1} (400 μmol kg^{-1}) capsaicin divided into three doses of 25 and 50 mg kg^{-1} on the first, and 50 mg kg^{-1} on the second day 10 to 16 days before the experiment according to the protocol of Gamse *et al.* (1981). For the part of the study testing the effect of vagotomy, all control rats were treated with equal volumes of the vehicle consisting of 10% ethanol and 10% Tween 80 in 154 mM NaCl (v/v). All injections were performed under ether anaesthesia.

Evaluation of results

Andrews & Lawes (1985) reported that gastric motor responses to extrinsic neural stimuli depended on the basal tone. The peak of the contractions, however, corresponded to the strength of the stimulus. Therefore the peak pressure reached during substance infusion, or within 1 min after the injection, was measured.

Response maxima, ED_{50}s, and slope factors were calculated for individual DRCs by fitting the data to sigmoid curves by use of the software ALLFIT (for description see De Lean *et al.*, 1978). All results are represented as mean ± s.e.mean. Since tests for normal distribution are not reliable with low n, statistical comparisons were made by the Kruskal-Wallis H-test (Sachs, 1982). A value of $P < 0.05$ was considered statistically significant.

Substances

SP, SP-methylester, and NKA were obtained from Cambridge Research Biochemicals (Cambridge, UK), bethanechol-Cl from Schuchardt (München, Germany), and phosphoramidon from Peninsula (St. Helens, UK). Captopril was kindly supplied by Squibb-von Heyden (Vienna, Austria). Stock solutions of the substances (1 mM for the peptides) as well as dilutions were made with 154 mM NaCl.

Results

During filling of the stomach with 3 ml saline, intragastric pressure increased sharply and then decreased again over several minutes to a level slightly higher than in the empty stomach reflecting adaptive relaxation. This rise in intragastric pressure was significantly greater in vagotomized rats than in rats with intact vagus or in ganglionectomized rats indicating impairment of adaptive relaxation (Table 1). Capsaicin pretreatment without vagotomy also caused a slight but significant augmentation of this rise in intragastric pressure indicating minor inhibition of adaptive relaxation. Conversely, vagotomy in capsaicin-pretreated rats produced a smaller rise in the intragastric pressure than vagotomy in vehicle controls (Table 1).

Retrograde injections of SP, NKA, or bethanechol into the splenic artery led to rapid and transient gastric contractions. After higher doses of NKA and bethanechol these tonic responses were followed by phasic activity lasting up to 10 min (cf. Holzer-Petsche *et al.*, 1987). The order of potency was NKA > SP > bethanechol (Table 2). There was no significant difference between the response maxima of SP, NKA, and bethanechol in control rats. The DRC for SP was significantly flatter than those for NKA and bethanechol (Table 2). SP-methylester in a dose range of 0.15–50 nmol induced only minor tonic gastric contractions up to 2.2 mN, which did not reasonably fit to sigmoid curves.

Captopril, whether injected into the splenic artery or i.v. (each $n = 4$) did not change the gastric motor response to a test dose of 0.5 nmol SP, although 30 min after i.v. captopril, mean blood pressure was significantly lowered from 98 ± 3 to 74 ± 2 mmHg. Phosphoramidon close arterially or i.v. (each $n = 3$) had no effect on either the gastric motor response to 0.5 nmol SP or on blood pressure.

Acute removal of the coeliac-superior mesenteric ganglion complex did not significantly influence gastric contractions in response to intraarterial infusions of NKA or SP into the coeliac artery (Figure 1). The calculated ED_{50}s were 0.22 ± 0.06 nmol for NKA and 15.8 ± 4.6 nmol for SP in control rats (each $n = 4$), 0.20 ± 0.03 nmol for NKA and

Table 1 Increase in baseline pressure (in kPa) 5 min after instillation of 3 ml saline into the stomach

Treatment	*Increase* (kPa)
Controls (untreated)	0.20 ± 0.08 (4)
Ganglionectomy	0.19 ± 0.03 (5)
Controls (vehicle-treated)	0.20 ± 0.02 (18)
Vagotomy (vehicle-treated)	0.63 ± 0.04 (17)[a]
Capsaicin	0.26 ± 0.02 (16)[c]
Capsaicin plus vagotomy	0.51 ± 0.03 (17)[a,b]

Values are means ± s.e.mean, (n).
[a] $P < 0.001$ vs. controls and capsaicin; [b] $P < 0.05$ vs. vagotomy; [c] $P < 0.05$ vs. controls and $P < 0.001$ vs. vagotomy and capsaicin plus vagotomy (Kruskal-Wallis H-test).

Table 2 Response maxima, ED_{50}s, and slope factors for the contractile effects of neurokinin A (NKA), substance P (SP) and bethanechol in control rats (treated with the vehicle for capsaicin) as obtained by fitting the experimental values to sigmoid curves by ALLFIT

	Maximum (kPa)	ED_{50} (nmol)	*Slope factor*[1]
NKA	4.66 ± 0.57	1.11 ± 0.47[a]	−0.96 ± 0.10
SP	5.64 ± 0.79	9.99 ± 3.62[a]	−0.48 ± 0.08[b]
Bethanechol	4.87 ± 0.23	39.4 ± 4.9	−0.92 ± 0.23

Common response minima were assumed.
Values are means ± s.e.mean, (n = 5–6).
[a] $P < 0.001$ vs. other two compounds; [b] $P < 0.05$ vs. NKA and bethanechol (Kruskal-Wallis H-test).
[1] 'The slope factor corresponds to the slope of the logit-log plot when the dose is portrayed in terms of natural logarithms' (DeLean *et al.*, 1978). Due to the assignment of the variables in the four-parameter logistic equation used by ALLFIT the slope factor is negative when the curve rises.

15.3 ± 0.6 nmol for SP in ganglionectomized rats (each n = 5). The slope factors were −0.59 ± 0.05 (n = 4) for NKA in controls, −0.80 ± 0.25 (n = 5) for NKA in ganglionectomized rats; −0.43 ± 0.15 (n = 4) for SP in controls, −0.46 ± 0.33 for SP (n = 5) in ganglionectomized rats (no statistically significant difference).

In vehicle-treated rats, the DRCs for NKA, SP, and bethanechol were shifted to the left by acute subdiaphragmatic vagotomy (Figure 2 and Table 3). Capsaicin pretreatment had no significant effect on the gastric responses to exogenous NKA or SP as long as the vagus nerves were intact. The DRC to bethanechol, however, was shifted to the left by capsaicin pretreatment. In rats pretreated with capsaicin, vagotomy induced only a minor shift to the left of the DRC for NKA (Figure 2a). The DRCs for bethanechol in vagotomized rats were identical whether the rats had been pretreated with capsaicin or its vehicle (Figure 2c). The leftward shift of the DRC for SP observed after vagotomy was reduced by capsaicin pretreatment (Figure 2b). Analysis of the DRCs by ALLFIT indicated no significant changes of the response minima, maxima, or slope factors.

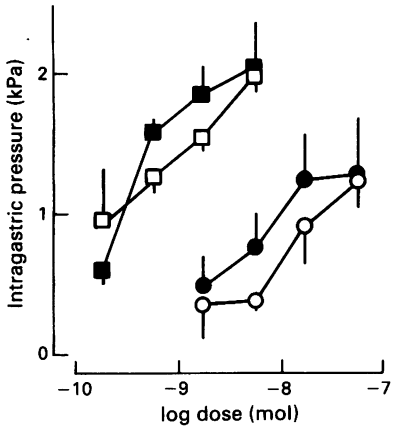

Figure 1 Effect of acute removal of the coeliac-superior mesenteric ganglion complex on gastric contractions induced by a 3 minute-infusion of substance P (○, ●) or neurokinin A (□, ■) into the coeliac artery; (○, □): controls; (●, ■): ganglionectomy. Values are means with s.e.mean shown by vertical lines; n = 4–5.

Table 3 Effect of various pretreatments on the relative potencies of neurokinin A (NKA), substance P (SP) and bethanechol as calculated from the ED_{50}s

	NKA	*SP*	*Bethanechol*
Vehicle	1 (1.11 nmol)	1 (9.99 nmol)	1 (39.4 nmol)[a]
Vehicle + vagotomy	17.5[a]	9.34[b]	12.0
Capsaicin	0.34	2.39	2.86
Capsaicin + vagotomy	2.30	7.62	11.3

[a] $P < 0.05$ vs. other three treatments.
[b] $P < 0.05$ vs. capsaicin and vehicle (Kruskal-Wallis H-test).

Discussion

The order of potency of the test substances for inducing gastric contractions in control rats confirms previous observations (Holzer-Petsche *et al.*, 1987). While the maximal responses to NKA, SP, and bethanechol were equal, the slope of the DRC for SP was smaller than those for the other two substances. The minor effect of SP-methylester indicates that NK_1 receptors contribute little to the gastric contraction induced by SP. SP itself is less specific for NK_1 receptors than SP-methylester, as higher doses also activate NK_2 receptors (Cascieri *et al.*, 1985), which is reflected by the greater ED_{50} of SP than NKA in control rats. Thus, the DRC for SP may represent activation of a mixed receptor population, a further indication of which is the smaller slope of the DRC for SP compared with NKA or bethanechol.

It has frequently been reported that neutral endopeptidase ('enkephalinase', EC 3.4.24.11) or peptidyl dipeptidase A ('angiotensin-converting enzyme', EC 3.4.15.1) is involved in the breakdown of SP (see Bunnett, 1987). Phosphoramidon, an inhibitor of neutral endopeptidase, impairs the breakdown of SP in the ferret small intestine (Djokic *et al.*, 1989), and peptidyl dipeptidase A appears to be involved in the breakdown of SP in the rat stomach wall as demonstrated by the use of captopril as inhibitor (Orloff *et al.*, 1986). Neither of the two inhibitors, however, had any effect on the gastric motor response to SP indicating that the faster breakdown of SP compared with NKA cannot be made responsible for the lesser potency of SP after close arterial injection.

Coeliac ganglionectomy did not increase gastric responses to NKA or SP. In contrast, pretreatment of the rats with guanethidine shifted the DRCs to both NKA and SP to the left, which was interpreted as abolition of an adrenergic inhibitory tone (Holzer-Petsche *et al.*, 1987). If such an adrenergic tone were exerted exclusively by splanchnic fibres, one would

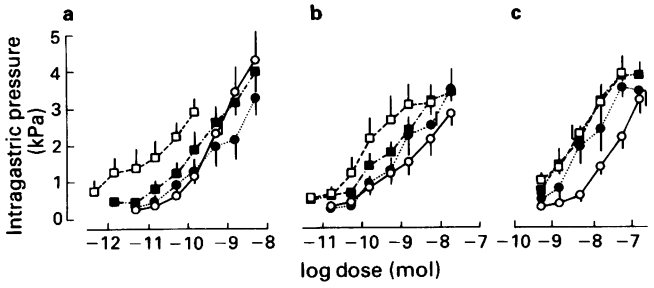

Figure 2 Dose-response curves for the gastric motor effects of neurokinin A (a), substance P (b), and bethanechol (c) after various pretreatments: (○) controls (vehicle-treated); (□) vagotomy (vehicle-treated); (●) capsaicin pretreatment; (■) capsaicin plus vagotomy. Values are means with s.e.mean shown by vertical lines; n = 5–6.

expect ganglionectomy to mimic guanethidine in stimulating the gastric responses to the tachykinins. The lack of effect of ganglionectomy, however, cannot be explained as being due to incomplete removal of the ganglia, since Kirchgessner & Gershon (1989) reported that the vagus nerve of rats contained postganglionic sympathetic axons from the superior cervical ganglion. Thus, sympathetic inhibition is not totally abolished after coeliac ganglionectomy but may be prevented by chemical sympathectomy.

Vagogomy significantly increased the potency of NKA, SP, and bethanechol in contracting the rat stomach. This suggests that an inhibitory influence is exerted via the vagus nerves, which, in rats with intact vagus, partly counteracts the evoked contractions. Vagal efferent discharge is modulated by changes in gastric wall tension, be they contraction, distension or compression of the stomach wall (Davison & Grundy, 1978). Thus it is feasible that every contraction of the stomach elicits a negative feedback via the vagus, which in turn counteracts the contraction. In the whole stomach, the measured change in gastric tension would therefore be the resultant of the contraction elicited by the agonist and the reflex relaxation. The increased contraction of the stomach in response to i.a. administered agents observed after vagotomy may thus be explained by the destruction of such a vagal negative feedback mechanism.

SP has been described as stimulating visceral afferents (Lew & Longhurst, 1986), and Maggi *et al.* (1986) proposed that, in the rat bladder, SP-E (NK_2) receptors (where NKA is more potent than SP) might be located on afferent nerve endings. In contrast, Cervero & Sharkey (1988) concluded from their experiments on afferents of rat ileum that the afferent stimulation by SP might be indirectly mediated through the peptide-induced contractions of the smooth muscle. To differentiate between these possibilities, bethanechol was also administered since electrophysiological experiments had demonstrated that this muscarinic agonist did not stimulate visceral or cutaneous afferent nerve fibres (Armett & Ritchie, 1961; Juan, 1982). The potentiation of bethanechol-induced gastric contractions by vagotomy suggests that the postulated negative feedback was initiated by the gastric contractions rather than by chemical stimulation of afferent nerve endings.

A tonic inhibition of the stomach via the vagus nerve has also to be considered as opposed to a reflex relaxation. However, since capsaicin pretreatment shifted the DRC for bethanechol to the left it seems that abolition of afferent nerve activity rather than of tonic efferent inhibition is responsible for the increased response to bethanechol after vagotomy. From the shift to the left of the DRCs for bethanechol after ablation of capsaicin-sensitive afferents it must be concluded that at least part of the mechanoreceptors in the gastric wall are sensitive to capsaicin and that these capsaicin-sensitive afferents are involved in the vago-vagal inhibition. This observation is paralleled by the slightly but significantly greater rise in baseline pressure after initial instillation of saline into the stomach of capsaicin-pretreated rats.

Unlike the DRCs for bethanechol in capsaicin-pretreated rats, which mimicked those after vagotomy in vehicle-treated rats, the DRCs for NKA in capsaicin-pretreated rats were similar to those in control rats whether or not the vagus nerves were intact. Explanation of this finding is possible by assuming that NKA initiates a positive feedback which uses capsaicin-sensitive structures and which does not require an intact vagus nerve. The negative feedback (via the vagus) and the positive feedback (via capsaicin-sensitive afferents) balance each other so that abolition of both regulatory mechanisms at the same time (as in capsaicin-pretreated rats with vagotomy) does not shift the DRC for NKA from its position in control rats.

The localization of the capsaicin-sensitive endings stimulated chemically by NKA cannot be deduced from the present experiments. If the positive feedback required impulse conduction through extrinsic centres, these afferents could not run in the vagus, because then they would also be destroyed by vagotomy in vehicle-treated rats. If the positive feedback used impulse conduction via the splanchnic nerves through the coeliac ganglion, it would be abolished after prevertebral ganglionectomy. Another mechanism to be considered for the processing of the positive feedback is a local one within the gastric wall. In this case the afferent nerve endings stimulated by NKA would mediate the positive feedback by means of an axon or enteric reflex. Neuhuber (1987) described afferent nerve endings in the proximal stomach of the rat which showed presynaptic structures in contact with intrinsic neurones and which might be the anatomical correlate of such an intramural reflex. Abolition of an intramural reflex involving capsaicin-sensitive afferents might explain the smaller increase in intragastric pressure after filling the stomach in the 'capsaicin plus vagotomy' group as compared to vehicle-treated rats with vagotomy (Table 1). If capsaicin-sensitive fibres are stimulated by changes in gastric tension, this stimulus might lead to a local positive feedback similar to the one postulated to be evoked by NKA. Such an intramural reflex would not be affected by acute vagotomy, since this procedure does not immediately impair the function of the peripheral endings of the cut nerve.

The shift of the DRCs for SP by the various treatments was less pronounced than for NKA. Since, however, SP acts only partly via muscular tachykinin receptors but also via cholinergic interneurones through atropine-sensitive receptors (Holzer-Petsche *et al.*, 1987), the mechanism of action of SP can be understood to comprise the actions of both NKA and bethanechol.

The present results show that extrinsic nerves are able to influence contractions induced by gastric motor stimulants. Vago-vagal inhibition of gastric motility is not only elicited by gastric distension but seems also to play a role in modulating drug-induced contractions. Capsaicin-sensitive afferents take part in mediating this modulation. Furthermore, there is indirect evidence for tachykinin receptors (presumably NK_2) on capsaicin-sensitive afferent nerve endings.

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A receptor that is highly specific for extracellular ATP in developing chick skeletal muscle *in vitro*

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1 Extracellular adenosine 5'-triphosphate (ATP) activated an early excitatory conductance followed by a late potassium conductance in developing chick skeletal muscle. A series of ATP analogues were tested for their ability to activate these two conductances. All compounds tested were either agonists for both responses or for neither. Furthermore, the potency of agonists was similar for the two responses.

2 The order of potency for agonists was ATP ≈ adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S) ≈ 2-methylthio-ATP (2-CH_3S-ATP) > 2'-deoxy-ATP ≈ 3'-deoxy-ATP > adenosine 5'-tetraphosphate (ATP-OPO_3) ≈ adenosine 5'-diphosphate (ADP). Many other ATP analogues were not agonists.

3 Activation of the excitatory response did not require divalent cations. Furthermore, the concentration-response relation of the excitatory response was similar when ATP was applied as the free anion of ATP (ATP^{4-}) or complexed with a divalent cation ($M \cdot ATP^{2-}$).

4 Three antagonists of the ATP response were characterized. 8-Br-ATP was a weak antagonist, while 2',3'-dialdehyde-ATP and DIDS (4,4'-diisocyanatostilbene-2,2'-disulphonic acid) were potent irreversible inhibitors. The two conductances were equally affected by these antagonists.

5 These results suggest that both ATP responses are activated through the same receptor type, or two very similar receptors.

Keywords: ATP; receptor; chick skeletal muscle

Introduction

Extracellular adenosine 5'-triphosphate (ATP) is known to activate a variety of cellular responses in many different tissues (Gorden, 1986). For example, there is good evidence suggesting that ATP participates in synaptic transmission from autonomic neurones to smooth muscle cells (Burnstock, 1981). ATP is packaged in cholinergic vesicles (Zimmermann, 1982), and released upon stimulation of motor neurones (Silinsky & Hubbard, 1973), so ATP receptors could potentially play a role in synaptic transmission at the skeletal neuromuscular junction. In developing chick skeletal muscle in culture, micromolar concentrations of extracellular ATP activate an early excitatory conductance followed by a late potassium conductance (Hume & Thomas, 1988). To test whether these two responses are activated by different receptor types, a variety of ATP analogues were screened for their ability to activate or inhibit these two responses, and the potencies of those analogues that were agonists were compared. This study also allowed us to evaluate systematically the effect of various alterations to the structure of ATP on the ability to activate responses. Our results are consistent with the possibility that both ATP responses are activated by the same receptor type. In addition, two antagonists which may prove useful in determining the function of the ATP receptor and in identifying the macromolecule(s) that constitute this receptor have been characterized.

Methods

Cell culture

Standard chick embryo muscle cell cultures were prepared as described previously (Hume & Honig, 1986). Briefly, pectoral muscle was dissected from 11 day old chick embryos, minced, and incubated in a calcium- and magnesium-free saline (Puck's saline) for 20 min at room temperature. The tissue was then spun down for 5 min, resuspended in culture medium, and triturated until the solution was cloudy. Cell density was determined with a haemocytometer after the suspension was filtered through lens paper to remove debris. To prepare myotube cultures, cells were plated onto gelatin-coated tissue culture dishes (Corning) at 150,000 per 35 mm dish. Myoballs (spherical, multinucleate muscle cells) were made in an identical manner up to the cell plating stage. Cells were preplated onto uncoated tissue culture dishes at 750,000 per 35 mm dish and incubated at 37°C for 2 to 3 h. This procedure greatly reduced the number of fibroblasts, which adhere more rapidly to the dishes than the muscle precursor cells. The culture dishes were then swirled several times and the medium of each dish (containing an enriched population of myoblasts) was transferred to a fresh, uncoated tissue culture dish. The high cell density promoted the formation of clusters of muscle precursor cells. The reduction in fibroblasts and the use of uncoated dishes made the culture dish surface poorly adhesive, which inhibited muscle cell elongation. Recordings were made from myotubes after 6 to 10 days in culture, or from myoballs after 3 to 6 days in culture.

The culture medium, Eagle's minimum essential medium (MEM) with Earle's salts (Gibco), was supplemented with 10% heat-inactivated horse serum (Gibco), penicillin/streptomycin (50 units ml^{-1}, 50 $\mu g\,ml^{-1}$) and conalbumin (40 $\mu g\,ml^{-1}$, Sigma). Cultures were maintained in a humidified incubator at 37°C with an atmosphere of 95% air, 5% CO_2.

Solutions

Recording was performed over a period of up to several hours at room temperature (21–23°C) without perfusion of the bath. Just prior to recording, each culture dish was washed at least three times over a period of 5 min with the appropriate external solution (Table 1) to replace the incubating media. Each wash exchanged about 3 ml. All solutions contained HEPES (as buffer) and 30 μM phenol red (as indicator) in order to maintain the pH near 7.3. NaOH and KOH were used to adjust the external and internal solutions respectively to pH 7.3.

The standard external solution contained cobalt, rather than calcium, to prevent the secondary activation of the calcium activated chloride current that is present in these cells (Hume & Thomas, 1989). External solutions containing ethylenediamine tetraacetate (EDTA) or ethyleneglycol-*bis*-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) were

Table 1 Composition of solutions (in mM)

Compound	Solution: Standard external	EDTA external	EGTA external	Internal
Na acetate	110	110	90	—
KCl	—	—	—	100
K acetate	4	4	4	—
TEA Cl	20	20	20	—
$CoCl_2$	4	—	—	—
$MgCl_2$	1	—	1, 3, or 9	2
Na_2H_2EDTA	—	5	—	—
Na_2H_2EGTA	—	—	11	—
K_4BAPTA	—	—	—	20
HEPES	12.5	12.5	12.5	10
Phenol red	0.03	0.03	0.03	0.03
Glucose	10	10	10	30

used to examine the role of extracellular magnesium. The EDTA solution was used to produce a solution essentially free of magnesium, while the EGTA solution was used to set the free magnesium concentration at different levels. The fraction of total ATP that was not complexed with Mg^{2+} was calculated from the equation:

$$ATP^{4-} = 1 - 1/(10^{x}*[Mg_{free}])$$

where x = 4.2, the association constant of $MgATP^{2-}$ under these conditions, and Mg_{free} was calculated assuming a K_d for Mg^{2+} of 300 μM (Walaas, 1958; Cockroft & Gomperts, 1979).

Intracellular and patch-clamp recording

Intracellular recordings were made with conventional glass microelectrodes filled with 3 M KCl as described previously (Hume & Honig, 1986). The high input resistance of myotubes bathed in the external solution allowed the membrane potential to be varied between +20 and −100 mV by passing very small currents (< 1 nA). The resistance of electrodes was nearly constant when such small currents were passed; thus a single microelectrode with a balanced bridge circuit was used both to record voltage and pass current. The bridge circuit was balanced just prior to penetration of each cell.

Standard techniques were employed to form high resistance seals with pipettes onto the membrane of myoballs and to gain access to the cell interior (Hamill *et al.*, 1981). Myoballs were dialyzed with the internal solution and bathed in one of the external solutions (Table 1). Polished pipettes had resistances of 2 to 4 MΩ, and recordings were made from myoballs whose diameters ranged from 15 to 30 μm.

Drugs

All compounds came from Sigma except for 2-methylthio-ATP (2-CH_3-S-ATP) (Research Biochemicals), adenosine 5′-O-(3-thiotriphosphate) (ATP-γ-S) and β,γ-imido-ATP (AMP-PNP) (Boehringer Mannheim). Agonists were dissolved in the appropriate external solution and loaded into pipettes having tip diameters of 2–4 μm. Solution was ejected from the pipettes by pressure applied to the back of the pipette. The duration of the pressure pulse was accurately controlled by a solenoid valve in the pressure line. When the solenoid closed, it vented the pipette to the outside, so that no residual pressure could continue to force drug from the pipette. The tip of the pipette was placed approximately 30–60 μm from the test cell. The compounds used in this study, and the abbreviations used were as follows: adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), inosine 5′-triphosphate (ITP), guanosine 5′-triphosphate (GTP), uridine 5′-triphosphate (UTP), cytidine 5′-triphosphate (CTP), 2-methylthio-ATP (2-CH_3-S-ATP), 8-bromo-ATP (8-Br-ATP), 2′- and 3′-O-(4-benzoylbenzoyl)-ATP (2′/3′-BB-ATP), adenosine 5′-tetraphosphate (ATP-OPO_3), adenosine 5′-O-(3-thiotriphosphate) (ATP-γ-S), α,β-methylene-ATP (AMP-CPP), β,γ-methylene-ATP (AMP-PCP), β,γ-imido-ATP (AMP-PNP), adenosine 5′-O-(3-thiotriphosphate) (ADP-β-S), adenosine 5′-diphosphoramidate (ADP-β-NH_2), adenosine 5′-diphosphomorpholidate (ADP-β-morpholidate), adenosine 5′-monophosphomorpholidate (AMP-α-morpholidate), adenosine 5′-phosphosulphate (AMP-OSO_3), adenosine 5′-monophosphoramidate (AMP-α-NH_2), P^1,P^2-di(adenosine 5′)pyrophosphate (A-P_2-A), P^1,P^3-di(adenosine 5′)triphosphate (A-P_3-A), P^1,P^4-di(adenosine 5′)tetraphosphate (A-P_4-A), P^1,P^5-di(adenosine 5′)pentaphosphate (A-P_5-A), P^1,P^6-di(adenosine 5′)hexaphosphate (A-P_6-A), 2′,3′-dialdehyde-ATP (oxidized ATP), 2′,3′-acyclic dialcohol-ATP (reduced ATP), 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (SITS).

Measurement of ATP and analogue responsiveness

The responsiveness to ATP was measured in one of two ways. Intracellular recordings from myotubes were used when screening ATP analogues because many cells could be quickly studied with this technique. Cells were initially adjusted to −70 mV, and the input resistance (R_{in}) was measured with a 0.1 nA hyperpolarizing current pulse. The peak depolarization activated by a 1 s application of an agonist was measured, and then divided by R_{in} to obtain an estimate for the current activated. For all agonists that activated an excitatory response, a semi-quantitative concentration-response curve was prepared by applying several different concentrations of agonist to a series of myotubes. This method was quick, but produced only a rough estimate of the parameters of the concentration-response curve, because large responses are subject to considerable non-linear summation. For this reason, EC_{50}s obtained by this method are reported only by their approximate magnitude (Table 2), rather than as discrete values.

Quantitative estimates of ATP responsiveness were obtained from whole-cell recordings made from cultured myoballs, since the ATP-evoked currents could be measured directly. Myoballs were voltage-clamped at either −80 mV for measurement of the excitatory ATP-activated current, or at +10 mV for measurement of the potassium current activated by ATP (see Figure 1). Cell capacitance was measured to obtain an estimate for the surface area of the cell. The peak current activated by a 1 s application of an agonist was measured, and then normalized to 100 pF of cell capacitance.

To obtain concentration-response curves, it was necessary to record from a population of cells for each concentration of each agonist. Data from 5 to 20 cells were averaged for each concentration, and then at least four different concentrations were studied for each agonist. It was not possible to obtain concentration-response curves from individual cells because both the excitatory and potassium currents show long-term desensitization to repeated applications of ATP (Thomas &

Hume, 1990b). A sigmoid curve was fitted to the averaged data by a least-squares non-linear regression programme (SigmaPlot 4.0, Jandel Scientific). The bottom of the sigmoid curve was held constant at 0, while the maximum, the EC_{50}, and the Hill slope were allowed to vary. To test whether a single curve could describe the relationship between agonist concentration and the amplitude of both ATP responses, the method described by Motulsky & Ransnas (1987) was used. After the data for the two responses to each agonist had been individually fitted, the two data sets were pooled (after dividing each individual response by the estimated maximal response), and the F ratio calculated. The average curve was considered to be an adequate fit to both data sets if P was >0.05.

Results

Low micromolar concentrations of extracellular ATP had a dual effect on developing chick skeletal muscle (Figure 1). ATP caused excitation by activating a class of small conductance (0.3 pS) ion channels through which both cations and anions permeate (Thomas & Hume, 1990a). ATP also elicited a potassium conductance that had a longer latency to activation than the excitatory conductance (Hume & Thomas, 1988). Concentration-response curves for the two currents activated by ATP are shown in Figure 2. A single curve fitted the data for both the excitatory and potassium responses.

To characterize the pharmacological specificity of the ATP receptor that elicited the excitatory current, ATP analogues substituted at several different positions were tested. The structure of ATP, with key positions labelled, is shown in Figure 3. Each analogue was tested with intracellular recording, by applying the substance at 0.5 or 1 mM to myotubes set

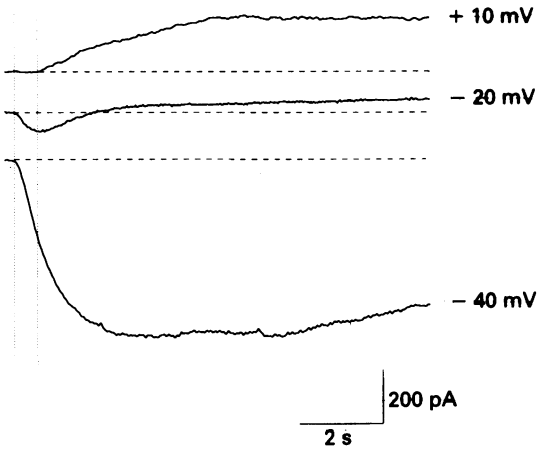

Figure 1 Activation of two distinct currents in chick skeletal muscle by ATP. Application of 10 μM ATP began at the time indicated by the first dotted line, and continued throughout the traces. For each trace, a horizontal dashed line indicates the holding current prior to the application of ATP. When a myoball was held at −40 mV (bottom trace), a large inward current was elicited with a delay of only a few milliseconds. After several seconds the current began to return towards the baseline. This return to baseline represented the activation of a second current. The presence of the second current could be seen more clearly when myoballs were held at more positive potentials. At −20 mV (middle trace) a biphasic current response was observed; the net current elicited by ATP was first inward and then outward. When a myoball was held at +10 mV (top trace), the early phase of the ATP response was absent, because the cell was at its reversal potential. ATP elicited only an outward current with a delay of several hundred milliseconds between the beginning of ATP application and the beginning of the response (indicated by second dotted line). At potentials positive to +10 mV (not shown), ATP evoked outward currents within a few milliseconds of application.

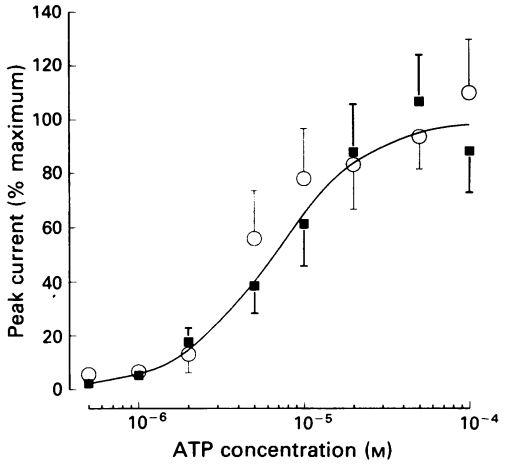

Figure 2 Concentration-response curves for the two currents activated by ATP. The peak current was measured in response to application of ATP from a nearby puffer pipette. Myoballs were voltage-clamped at −80 mV to isolate the excitatory current (■, thick error bars), or at +10 mV to isolate the potassium current (○, thin error bars). For each concentration of ATP, 5–20 cells were tested, and the ATP evoked currents were normalized (to pA/100 pF) and then averaged. To allow for presentation of concentration-response data for both responses on a single set of axes, least squares fits to the data for excitatory and potassium responses were performed separately. The average response and standard error for each concentration of ATP were divided by the maximal current indicated by the fitted curves. The solid line represents the least squares fit to the data for the excitatory response. The parameters of this curve were EC_{50} = 6.6 μM, Hill coefficient = 1.5, maximal current = −1092 pA. Except for the expected difference in the maximal current reached, the curve fitted to the potassium current data was not significantly different (EC_{50} = 5.1 μM, Hill coefficient = 1.6, maximal current = +773 pA).

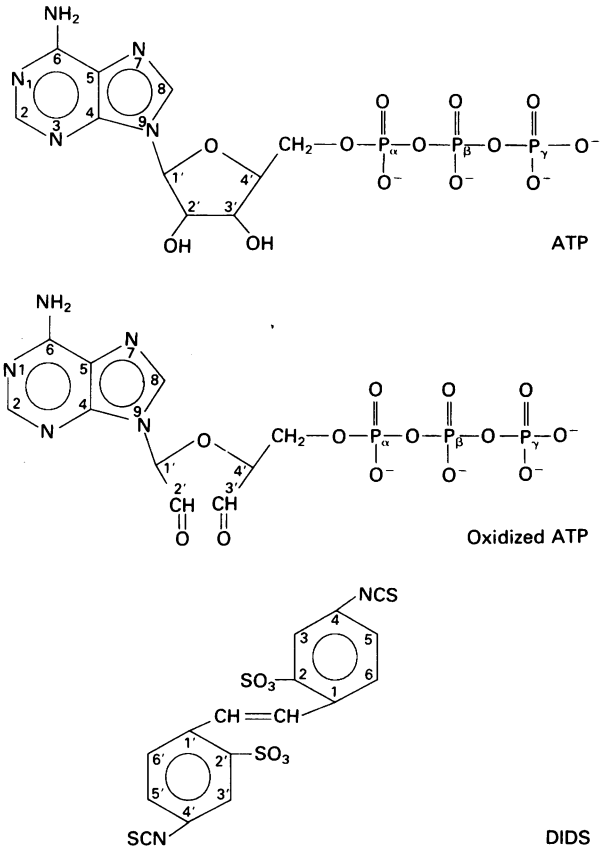

Figure 3 Primary structure of ATP, 2',3'-dialdehyde-ATP (oxidized ATP) and 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS).

to a potential of −70 mV. At least 10 fibres from two different platings were tested for each analogue. The ability of ATP analogues modified at various positions to elicit a depolarization is summarized in Table 2.

Twenty six of the ATP analogues that were tested for their ability to activate the excitatory response were also tested for their ability to activate the potassium current. In these experiments, myoballs were voltage clamped at +10 mV, the reversal potential for the excitatory response. The potassium current had a pharmacological spectrum very similar to that for the excitatory current; each of the fifteen analogues that elicited the excitatory current also activated the potassium current and each of the eleven analogues that failed to activate the excitatory current also failed to activate the potassium current. The concentration-response relationships for both responses were examined semi-quantitatively for all 15 agonists (Table 2) and quantitatively for 8 agonists covering a wide spectrum of potencies (Table 3). For all agonists, there was great similarity in the EC_{50} for the excitatory and potassium currents activated by ATP. For 6 of the 8 agonists examined in detail, statistical analysis indicated that a single, averaged curve could describe the concentration-response relationship for either response as well as the individual curves, but statistical analysis indicated that, at the 5% confidence level, the best curves describing the two responses elicited by ATP-γ-S and by 3'-deoxy-ATP were not the same. One interpretation of these results is that the excitatory and potassium currents are activated by two distinct, but similar receptors. However, this conclusion may be incorrect. In both of these cases, one of the curves had a Hill coefficient that, based on the data for the other agonists, was aberrant. If the fits were constrained to have a Hill coefficient between 1 and 2, as suggested by the bulk of the data for agonists of high potency, then the best fitted curves for these two agonists were no longer significantly different. For this reason, there is at present no compelling reason to postulate that more than a single class of ATP receptors is present on the surface of chick skeletal muscle cells.

Table 2 Activity of ATP analogues

Site altered	*Agonist potency*			*Inactive compounds*
	EC_{50} <50 μM	EC_{50} >50 μM and <500 μM	EC_{50} >500 μM	
Adenine	2-CH_3S-ATP!			8-Br-ATP*!
			N^1-oxide-ATP!	$1,N^6$-etheno-ATP
				ITP!; GTP!
				CTP!; UTP!
Ribose		2'-deoxy-ATP!		oxidized ATP!
		3'-deoxy-ATP!		reduced ATP!
		2'/3'-BB-ATP!		
Phosphate	ATP-γ-S!			ADP-β-S!
		ATP-OPO_3!		ADP-β-NH_2!
		ADP!		ADP-β-morpholidate*
			AMP-PNP!	ADP-ribose
			AMP-CPP!	ADP-glucose*
			AMP-PCP!	A-P_2-A
			A-P_3-A!	AMP!
			A-P_4-A!	AMP-α-NH_2
			A-P_5-A!	AMP-OSO_3
			A-P_6-A!	AMP-α-morpholidate*
				Adenosine!
Multiple				2'-deoxy-ADP*

To test whether an analogue was an agonist for the excitatory response, the analogue was pressure-ejected (at 1 mM, except where indicated by *, when 0.5 mM was used) from a pipette for 1 s while recording the membrane potential from a nearby myotube. The initial membrane potential of the myotube was adjusted to −70 mV prior to application of an analogue. The analogue was scored as an agonist if it produced a depolarization in several myotubes. For analogues that did not produce a response, we confirmed that the cells depolarized in response to 10 μM ATP. The potency of all agonists was estimated by use of intracellular recording methods. Because this method was only semi-quantitative, EC_{50}s are given only by their approximate magnitude, rather than as discrete values. The potency of a subset of agonists was also assessed quantitatively by whole-cell recording (Table 3). Compounds indicated with an ! were also tested for their ability to elicit potassium currents in myoballs voltage clamped to +10 mV. All compounds tested for activation of the potassium current fell in the same potency class as indicated for the excitatory responses. For abbreviations, see Methods.

Table 3 Potency of the agonists

	EC_{50} (μM)		*Hill slope*	
Agonist	*Excitatory*	*Potassium*	*Excitatory*	*Potassium*
ATP*	7	5	1.5	1.6
ATP-γ-S	5	11	3.4	2.2
2'-deoxy-ATP*	79	78	1.7	2.4
3'-deoxy-ATP	110	47	1.8	2.9
ATP-OPO_3*	62	188	2.1	0.8
ADP*	231	282	1.9	4.9
AMP-PNP*	3280	6380	0.8	0.5
AMP-CPP*	6620	5600	0.8	0.4

For each agonist, data for concentration-response curves were collected and analyzed as described in Methods. This table presents the parameters that best fitted the excitatory and potassium responses generated by each agonist. However, for 6 of the 8 agonists (indicated by *), statistical analysis indicated that a single curve could describe both the excitatory and potassium responses as well as the individual curves. For abbreviations, see Methods.

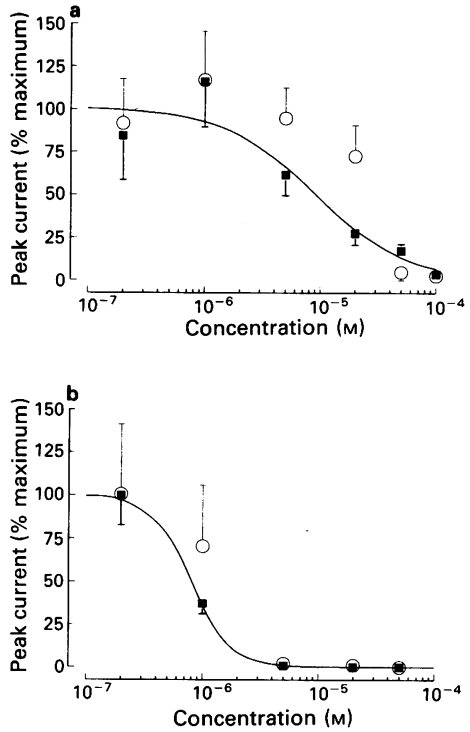

Figure 4 Inhibition of the two ATP responses by (a) 2',3'-dialdehyde-ATP (oxidized ATP) and (b) 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS). Myoballs were incubated in the external solution with the indicated concentration of inhibitor for 1 h at room temperature prior to recording. The peak current activated by 50 μM ATP (applied from a pipette that did not contain the antagonist) was measured. The cells were voltage-clamped at −80 mV to study the excitatory current (■, thick error bars) or at +10 mV to study the potassium current (○, thin error bars). The smooth curves represent the curve fit to the data for the excitatory current. For oxidized ATP (a), the best curve for inhibition of the excitatory response had an IC_{50} of 9.4 μM and a Hill slope of −1.3, while the best curve for inhibition of the potassium conductance had an IC_{50} of 25 μM and a Hill slope of −4.2. For DIDS (b), the best curve for inhibition of the excitatory response had an IC_{50} of 0.8 μM and a Hill slope of −2.6, while the best curve for inhibition of the potassium conductance had an IC_{50} of 1.3 μM and a Hill slope of −2.9. In neither case did the two curves differ significantly.

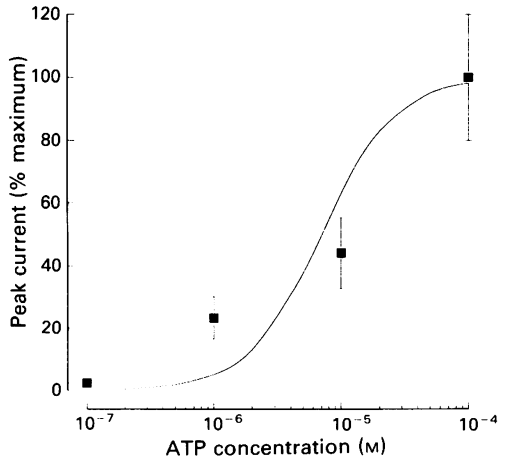

Figure 5 Activation of the excitatory response by ATP^{4-}. Myoballs were voltage clamped at −80 mV. The data points illustrated were taken from cells bathed in EDTA external, where virtually all the ATP was in the form of ATP^{4-}. The smooth curve is the one that was fitted to cells bathed in normal external solution, where virtually all the ATP was in the form of $M \cdot ATP^{2-}$. The curves were normalized to a single set of axes by assuming that the maximal current in these experiments was −295 pA/100 pF.

All analogues that were not agonists were tested as potential antagonists by adding the analogue (at 0.5 mM) to the external solution in the bath and to the solution in the pipette used to apply ATP. For these experiments, a concentration of 10 μM ATP was used, because it was in the steep region of the concentration-response curve, and thus would be most sensitive to antagonist activity.

Only two classes of ATP analogues were found to be antagonists. They were selective, since they did not inhibit the response of muscle cells to 10 μM acetylcholine. At a concentration of 0.5 mM, 8-Br-ATP reduced the response to 10 μM ATP and eliminated the response to 2 μM ATP. The triphosphate group was required for antagonism; neither 8-Br-ADP nor 8-Br-AMP were effective antagonists at 0.5 mM. Because it had to be present at high concentration to block the response to ATP, the antagonism by 8-Br-ATP was not analyzed in detail. A much more potent antagonist was the periodate-oxidized analogue of ATP: 2',3'-dialdehyde-ATP (oxidized ATP). Inhibition by this molecule was both time- and temperature-dependent. With 100 μM oxidized ATP, almost complete antagonism developed in about 30 min at room temperature, and it developed more rapidly at 37°C. Inhibition could not be overcome with higher concentrations of ATP, and block was still effective after washing out the external solution containing oxidized ATP.

Approximately half of either the excitatory or the potassium conductance activated by ATP could be blocked by 10 μM oxidized ATP (Figure 4).

Interestingly, although ADP and AMP were much less potent agonists than ATP, oxidized ADP and oxidized AMP were as potent as oxidized ATP at blocking the response to ATP. However, oxidized adenosine had no effect on the ATP response. In addition, the oxidized forms of a number of nucleotide triphosphates that had no agonist activity (ITP, GTP, UTP, and CTP) blocked the response to ATP in a similar manner. However, these compounds required more time to be effective. For example, when a 1 h incubation at room temperature was used, these compounds were less effective than oxidized ATP, but they were almost as effective as oxidized ATP after a 1 h incubation at 37°C.

McMillian *et al.* (1988) reported that the stilbene derivative DIDS (4,4'-diisothiocyanatostilbene 2,2'-disulphonic acid) was a selective blocker of the ATP-induced increase in intracellular free calcium in rat parotid acinar cells. In chick skeletal muscle, DIDS was an even more potent antagonist of the ATP response than any of the ATP analogues tested. DIDS was similar to oxidized ATP in that its block was both time- and temperature-dependent, and could not be overcome with higher concentrations of ATP. Complete block of both conductances activated by ATP was observed with 20 μM DIDS, while approximately 1 μM DIDS was required to block half of each response (Figure 4). The related stilbene derivative SITS (4-acetamido-4'-isothiocyanatostilbene 2,2'-disulphonic acid) was approximately ten fold less potent in blocking the ATP response.

Activation: ATP^{4-} versus $MgATP^{2-}$

In a solution which contains divalent cations, the free anion ATP^{4-} is in equilibrium with $M \cdot ATP^{2-}$, where M represents a divalent cation. One class of ATP receptors is known to be much more sensitive to ATP^{4-} than to $M \cdot ATP^{2-}$ (Cockroft & Gomperts, 1979). Two sets of experiments were performed to evaluate the relative importance of ATP^{4-} and $M \cdot ATP^{2-}$. In one set of experiments, the total concentration of ATP was kept constant at 5 μM, but the ratio of ATP^{4-} to $Mg \cdot ATP^{2-}$ was varied by adding differing amounts of magnesium to solutions buffered with 11 mM EGTA. For the concentrations of total magnesium used, the free magnesium concentration varied from 36 μM to almost 1 mM, which produced concentrations of ATP^{4-} from 0.3 to 3.2 μM. Over this ten fold range of ATP^{4-}, the magnitude of the responses to ATP (estimated from intracellular recordings made from

myotubes) remained essentially constant (0.3 μM, -312 ± 38 pA; 1.7 μM, -349 ± 91 pA; 3.2 μM, -235 ± 25 pA). This result indicated either that the receptor for the excitatory response does not distinguish between ATP^{4-} and $Mg \cdot ATP^{2-}$, or that 0.3 μM ATP^{4-} is a saturating concentration. To test the latter possibility, cells were exposed to a solution containing 5 mM EDTA, without adding any divalent cations. In this solution ATP is almost entirely in its free anion form, ATP^{4-}. When myoballs were bathed in this solution and held at -80 mV, inward currents were activated by ATP over a concentration-range similar to that in the standard external solution, and the concentration-response relationship was not significantly different from that for ATP in normal external solution (Figure 5). Thus the receptor for ATP does not appear to discriminate between ATP^{4-} and $M \cdot ATP^{2-}$, nor does it require divalent cations for activation.

Discussion

ATP activates an excitatory conductance and a potassium conductance in developing chick skeletal muscle (Hume & Thomas, 1988). In the work described here, a series of ATP analogues was tested for their ability to activate these two responses. We found that the same set of analogues activated each conductance. Furthermore, the concentration-response relationship for each agonist was similar for the two responses. These results are consistent with the possibility that the two responses are activated by one receptor type for ATP, rather than by two distinct receptor subtypes.

There are at least three mechanisms by which a single receptor class could activate two distinct responses. One possibility is that activation of the second response is a consequence of activation of the excitatory response. For instance, calcium ions might flow into the cells during the early response and activate a calcium-dependent potassium current. However, this particular explanation cannot be correct, since the potassium current can be evoked in the absence of extracellular calcium. Recordings from cell-attached patches and excised outside-out patches indicate that the potassium current is activated by a diffusible second messenger and suggest that this messenger is not responsible for activation of the excitatory response (Hume & Thomas, 1990). Therefore, a second possibility is that binding of ATP to its receptor produces two different second messengers, one capable of only limited diffusion, which activates the excitatory response, and a second that can diffuse longer distances to activate the potassium current. A third possibility is that the ATP receptor is part of a molecular complex that comprises both an ion channel and an enzymatic activity that produces the second messenger that activates the potassium current. This third scenario is consistent with the observation that the excitatory current can be activated within a few milliseconds of application of ATP (Hume & Thomas, 1988). Finally, it is possible that there are two distinct, but very similar ATP receptors, one of which is coupled to the excitatory channel and the other coupled to the production of a second messenger.

The specificity of ATP

The receptor for ATP is relatively specific. ADP was about 20 times less potent as an agonist while AMP and adenosine were ineffective. Other nucleotide triphosphates were unable to activate the responses to ATP. Thus most if not all stimulation of this receptor *in vivo* probably results from exposure to ATP. Since most of the agonists had Hill slopes between 1 and 2, it seems likely that the receptor must bind 2 molecules of ATP to be activated.

By comparing the potency of the various analogues, one can learn what sites of the ATP molecule are important for activation of the receptor. The effect of modifying the adenine base of ATP depended on the site of modification. At least several sites of the adenine moiety appear to be responsible for nucleotide-specific recognition. These include N^1 and the amino group of C^6. Oxidation of the N^1 nitrogen created a low potency agonist. Reaction of ethene with N^1 and the amino group of C^6, which created a third ring, eliminated agonist activity. ITP, which is identical to ATP except that the amino group of C^6 is replaced by a carbonyl oxygen, was also ineffective. The area around C^2 seems to be relatively unimportant since the addition of a methylthio group to this carbon did not reduce potency. Additions to C^2 force ATP to stay in the 'anti' conformation, in which the base points away from the phosphates so that the molecule is fully extended (as shown schematically in Figure 3). In contrast, the addition of bromide to C^8 forces ATP to adopt the 'syn' conformation almost exclusively (the base points in the direction of the phosphates, so the molecule looks like a U). This addition eliminated agonist activity and instead produced an antagonist, suggesting that the 'anti' conformation is required for activation.

There also appeared to be important interaction sites between the receptor and the ribose of ATP. Both 2'- and 3'-deoxy-ATP were ten fold less potent than ATP as agonists. Interestingly, when these two sites were oxidized to aldehydes, the analogue became a fairly potent irreversible antagonist. These results suggest that normally the hydroxyls of the ribose hydrogen-bond to sites in the receptor. These sites might react to form a covalent bond when brought next to an aldehyde. When the aldehydes of oxidized ATP were reduced to alcohols, no antagonism was observed, demonstrating the importance of these aldehydes to antagonism. The lack of specificity for adenine as the base of the oxidized nucleotide, and the requirement that the nucleotide be only a monophosphate to block the response, suggests that the minimal molecular unit for an antagonist might be simply oxidized ribose 5'-monophosphate.

With respect to activation, the length of the phosphate chain was critical. Adenosine tetraphosphate and ADP were approximately ten fold less potent than ATP, and AMP and adenosine were without effect. Of the agonists tested, all modifications to the β phosphorous of ADP eliminated agonist activity. This may have been for steric reasons when large groups such as glucose or adenosine were added. However, the lack of any agonist activity with ADP-β-S and ADP-β-NH_2 in contrast to ADP suggests that the oxygen atoms in the second phosphate group may be important for activation. On the other hand, ATP-γ-S was as potent an agonist as ATP, indicating that the oxygens of the third phosphate are less crucial than those of the second. The two oxygens linking the three phosphates of ATP together must be important, since replacing them with either carbon or nitrogen lowered agonist potency dramatically. The di-adenosine phosphates (with the exception of the diphosphate) were low potency agonists. As might be expected for two separate sites of interaction between the receptor and ATP, combining two modifications that partially reduced the potency resulted in an analogue that was inactive (2'-deoxy-ADP).

Classification of ATP receptors on skeletal muscle

Since ATP but not adenosine is an agonist, then by definition the receptors must be in the second class of purinoceptors (P_2) (Burnstock, 1981). P_2 receptors have been broken down into several subtypes: T, X, Y, Z. The T and Z receptors have been described in circulating and immune cells, while X and Y have been found in smooth muscle, endothelium, liver, pancreas and parotid acinar cells (Gorden, 1986). The receptors on chick skeletal muscle do not meet the established criteria for belonging to any of these classes. The criteria for assigning a receptor to the P_{2X} class are that 2-CH_3S-ATP is approximately equipotent to ATP, (as was observed in chick skeletal muscle) and that AMP-PNP and AMP-PCP are more potent than ATP (contrary to what was observed in chick skeletal

muscle). The criteria for assigning a receptor to the P_{2Y} or P_{2T} classes are that AMP-PNP and AMP-PCP are less potent than ATP (as was observed in chick skeletal muscle) and that 2-CH_3S-ATP is more potent than ATP (unlike chick skeletal muscle). In addition, the P_{2T} receptor responds to ADP as well as or better than ATP (unlike what was observed for chick skeletal muscle). Finally, the criteria for assigning a receptor to the P_{2Z} class are that the responses are largely or exclusively to ATP^{4-}, and that 2'/3'-BB-ATP is a more potent agonist than ATP. Both of these criteria are contrary to the results obtained for chick skeletal muscle.

The pharmacology of the chick skeletal muscle receptor appears to be most closely related to that described for cardiac muscle. ATP activates a small conductance cation channel in frog atrial myocytes (Friel & Bean, 1988), and increases intracellular free calcium in rat ventricular myocytes (De Young & Scarpa, 1987). The only pharmacological differences were that ADP was not effective at 100–200 μM, ATP-γ-S was slightly less potent than ATP, and AMP-CPP was an antagonist in frog cardiac muscle. ATP also activates a cation channel in arterial smooth muscle, but this receptor probably falls under the P_{2X} subtype since AMP-CPP was equipotent with ATP (Benham & Tsien, 1987).

ATP also elicits excitatory responses from several neuronal cell types. ATP activates cation channels in rat sensory neurones of spinal cord and brainstem ganglia (Krishtal *et al.*, 1983; 1988). These receptors have a similar pharmacology to that in chick skeletal muscle. However, for other neuronal ATP receptors the pharmacology is quite different. In the central nervous system, a subpopulation of dorsal horn neurones are excited by ATP. However, excitation is also elicited by AMP-PNP and AMP-PCP, suggesting that this receptor is of the P_{2X} subtype (Jahr & Jessel, 1983). A much less specific excitatory response is seen in bullfrog sympathetic neurones, where most nucleotide triphosphates are effective (Siggins *et al.*, 1977).

Finally, it has been shown that ATP stimulates the production of inositol phosphates in chick skeletal muscle (Haggblad & Heilbronn, 1987). However, the reported specificity of this response is different from what we have described for channel activation. Production of inositol phosphates is reported to be stimulated equally well by ATP and AMP-PNP, and less potently by AMP and adenosine. These results seem to suggest the possibility of two receptor subtypes for ATP in chick skeletal muscle. The alternative hypothesis is that activation of only a small number of receptors by AMP-PNP is sufficient to produce a significant increase in inositol phosphates, but not an electrical response. It would be interesting to test whether oxidized ATP or DIDS blocked the accumulation of inositol phosphates by ATP.

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Effect of N^G-monomethyl L-arginine (L-NMMA) and N^G-nitro L-arginine (L-NOARG) on non-adrenergic non-cholinergic relaxation in the circular muscle of the human ileum

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1 We have investigated the effect of the NO synthesis inhibitors, N^G-monomethyl L-arginine (L-NMMA) and N^G-nitro L-arginine (L-NOARG) on the non-adrenergic non-cholinergic (NANC) relaxation produced by electrical field stimulation in the circular muscle of the human ileum.

2 In the presence of atropine and guanethidine (1 and 3 μM, respectively), electrical field stimulation produced tetrodotoxin-sensitive relaxation of the strips. L-NMMA, starting from 100 μM, produced a concentration-dependent inhibition of the evoked relaxations (2 Hz); maximal inhibition at 1 mM averaged about 35%.

3 The inhibitory effect of L-NMMA was unchanged by previous incubation with D-arginine while it was prevented by L-arginine (L-Arg). L-NMMA did not affect isoprenaline-induced relaxation.

4 L-NOARG (1–30 μM) concentration-dependently inhibited the evoked relaxations at 2 Hz, up to a maximum of 65% inhibition, although in some strips complete inhibition of the response was observed. The effect of L-NOARG was reversed by L-Arg. L-NOARG did not affect isoprenaline-induced relaxation.

5 These results suggest that NO may be involved in inhibitory NANC transmission in the circular muscle of the human ileum.

Keywords: Human ileum; circular muscle; NO; NANC transmission; N^G monomethyl L-argine; N^G nitro L-arginine.

Introduction

The motility of the mammalian intestine is regulated by a complex network of neurones and nerve fibres of both intrinsic and extrinsic origin. Physiological experiments have established that in addition to cholinergic and non-cholinergic excitatory nerves, non-adrenergic non-cholinergic (NANC) nerves also exist in the gut which are thought to take part in descending relaxation and enable bolus progression in the caudal direction. The identity of the NANC transmitter(s) of the mammalian gut is still unresolved: both adenosine triphosphate (ATP) and vasoactive intestinal polypeptide (VIP) have been proposed as likely mediators in different species and/or different segments of the mammalian intestine (see McKirdy, 1988 for review). It has also been proposed that more than one NANC inhibitory transmitter exists in the mammalian gut and that different mediators play a different role in various intestinal segments of the same species (Costa *et al.*, 1986; Manzini *et al.*, 1986).

Nitric oxide (NO) generated from the guanidino nitrogen atom(s) or L-arginine (L-Arg), has been established as a mediator of the endothelium-dependent vasorelaxation (Palmer *et al.*, 1987; 1988). More recently, evidence has been presented indicating that NO could be an inhibitory transmitter in the mammalian intestine. Blockers of NO synthesis have been shown to reduce NANC inhibitory transmission in the canine ileocolonic junction (Boeckstaens *et al.*, 1990) and duodenal longitudinal muscle (Toda *et al.*, 1990), rat stomach (Li & Rand, 1990) and rat proximal colon (Hata *et al.*, 1990). Furthermore, recent evidence indicates the presence of NO synthase-like immunoreactivity in the myenteric plexus of the rat intestine (Bredt *et al.*, 1990). These findings have raised the question about a possible role of NO as a NANC inhibitory transmitter in the mammalian gut.

We have shown previously that inhibitory NANC nerves mediate the evoked relaxation to electrical field stimulation of the longitudinal and circular muscle of the human ileum (Maggi *et al.*, 1989; 1990). We describe here the effect of N^G-monomethyl L-arginine (L-NMMA) and N^G-nitro L-arginine (L-NOARG), two inhibitors of NO synthesis (Palmer *et al.*, 1988; Hobbs & Gibson, 1990), on the nerve-mediated NANC relaxation of the circular muscle of the human ileum.

Methods

Experiments were performed on mucosa-free circular muscle strips from the human ileum. Samples were obtained from patients undergoing enterocystoplasty for carcinoma of the urinary bladder ($n = 13$, age 62–79 years). Preanaesthetic medication, induction and maintenance of anaesthesia were as described previously (Maggi *et al.*, 1989; 1990). The excised samples were placed in oxygenated (96% O_2 and 4% CO_2) Krebs solution (pH 7.4 at 37°C).

Experiments were performed on either fresh or stored strips, as described previously. No major difference was observed in the behaviour of fresh and stored strips and the results were pooled. The composition of the Krebs solution was as follows (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. In all experiments the Krebs solution also contained atropine and guanethidine (1 and 3 μM respectively).

The strips were placed in 5 ml organ baths at 37°C and mechanical activity recorded by means of an isotonic transducer (load 0.5 mN). Electrical field stimulation was delivered by means of two wire platinum electrodes placed at the top and the bottom of the organ bath, connected to a GRASS S11 stimulator. Square wave pulses (0.5 ms pulse duration, maximal voltage) were delivered for 10 s at frequencies of 2, 5 or 10 Hz at 60–90 s intervals.

Relaxant responses were expressed as % of the maximal relaxation produced by isoprenaline (10 μM) and contractile responses as % of the maximal response to KCl (80 mM).

Each value in the text and Figure 2 is mean ± s.e.mean. Statistical analysis was performed by means of Student's t test for paired or unpaired data or by means of analysis of variance, when applicable.

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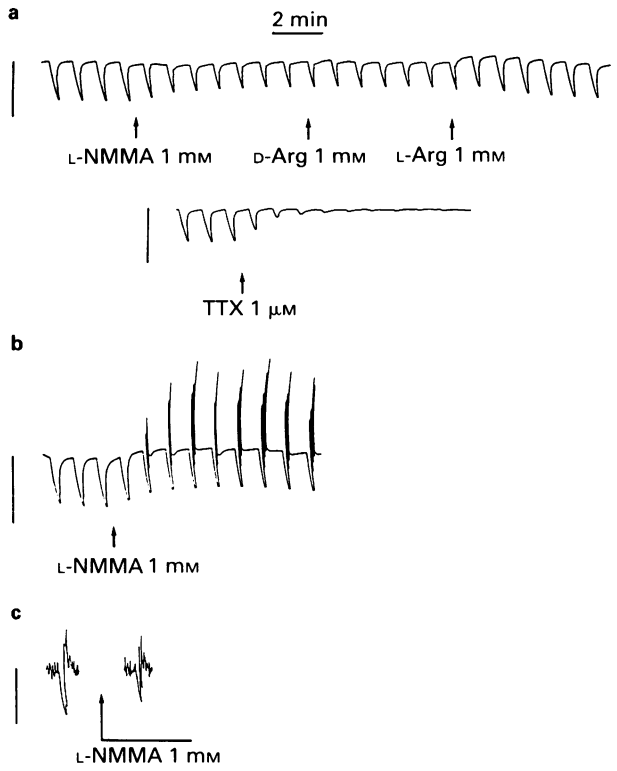

Figure 1 Original tracings showing the effect of N^G-monomethyl L-arginine (L-NMMA) on nerve-mediated (electrical field stimulation at 2 Hz) non-adrenergic non-cholinergic relaxation of the circular muscle of the human ileum, including reversal of L-NMMA action by L-arginine (L-Arg) but not by D-arginine (D-Arg) (a), blockade of evoked relaxation by tetrodotoxin (TTX, a, continued), and variable effects of L-NMMA on rebound contractions (b and c, see text for details). Vertical bars indicate maximal relaxation to isoprenaline.

Drugs used were: N^G-monomethyl L-arginine (L-NMMA) (Ultrafine Chemicals), N^G-nitro L-arginine (L-NOARG), D- and L-arginine, tetrodotoxin (Sigma), guanethidine sulphate (I.C.F.I.), atropine HCl and isoprenaline HCl (Serva).

Results

In the presence of atropine and guanethidine, electrical field stimulation of the circular muscle of the human ileum produced relaxation which was maximal at frequencies between 5 and 10 Hz, as found previously (Maggi *et al.*, 1990). The mean relaxant response observed in 8 strips was: 72 ± 6, 85 ± 5 and 81 ± 6% of the maximal relaxation to isoprenaline at 2, 5 and 10 Hz, respectively. A rebound contraction was observed in some instances at 2 Hz and in all instances at 10 Hz. At 10 Hz it ranged between 15 and 35% of the maximal contraction to KCl (80 mM). All the responses evoked by electrical field stimulation were abolished by tetrodotoxin (1 μM, $n = 4$, Figure 1).

The effect of L-NMMA on the relaxation produced by electrical field stimulation at 2 Hz was studied in 9 strips. At 10 and 30 μM, L-NMMA was without effect on the evoked relaxation. At 100, 300 and 1000 μM, L-NMMA produced a concentration-dependent inhibition of the evoked relaxation which averaged 14 ± 4, 29 ± 4 and 35 ± 3% of the response (Figure 2), respectively. L-NMMA had variable effects on the rebound contraction: in some instances it was unaffected, while in others it was unmasked (Figure 1b) or even slightly depressed (Figure 1c). These effects were concentration-independent. The effect of L-NMMA on relaxation produced by electrical stimulation at 10 Hz was also investigated ($n = 4$); at 1 mM, L-NMMA produced inhibition averaging 42 ± 5%.

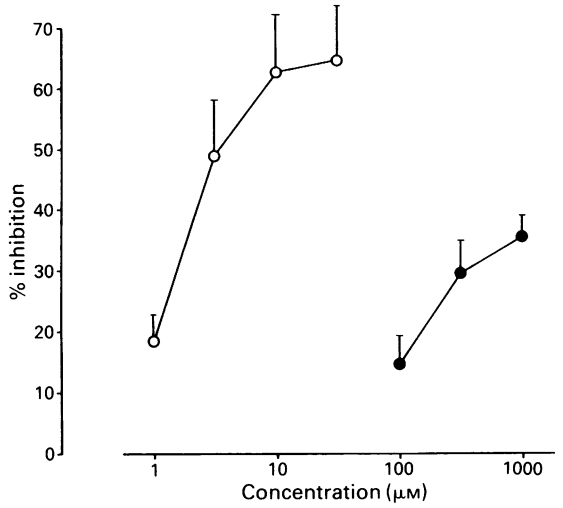

Figure 2 Concentration-dependent inhibition of evoked non-adrenergic non-cholinergic relaxations (electrical field stimulation at 2 Hz) of the circular muscle of the human ileum by N^G-monomethyl L-arginine (L-NMMA, ●) and N^G-nitro L-arginine (L-NOARG, ○). Each point is mean of 7–9 experiments; vertical lines show s.e.mean.

The effect of L-Arg or D-Arg on the L-NMMA-induced inhibition of evoked relaxation was also investigated. Neither amino acid (1 mM) had any significant effect on the evoked relaxation ($n = 4$ in each case). In strips pre-incubated with L-Arg (1 mM for 10 min), L-NMMA (1 mM) failed to reduce the evoked (2 Hz) relaxation while in strips pre-incubated with D-Arg (1 mM) it produces an inhibitory effect not different from that observed in controls. Similarly, inhibition produced by L-NMMA was reversed by addition of L-, but not D-Arg (Figure 1a).

The effects of L-NOARG were studied on the evoked relaxation at a frequency of 2 Hz in 7 strips. L-NOARG was much more potent and effective than L-NMMA in inhibiting the evoked relaxations; a significant reduction was observed even at 1 μM and a maximal effect at 30 μM (Figure 2). In individual strips the maximal inhibition produced by L-NOARG ranged between 33 and 100% (average 65 ± 12, Figure 2) which was reversed in 5–10 min by L-Arg: in fact, in the presence of 100 (Figure 3) and 300 μM L-Arg, the evoked relaxations recovered to about 70 and 90% of their original amplitude (before L-NOARG addition, $n = 5$). Examples of two strips responding to L-NOARG with total or partial inhibition of evoked relaxations are shown in Figure 3. L-NOARG also produced a concentration-dependent rise in tone of the tissue in 6 out of 7 strips which at 10 μM averaged 18 ± 4% of maximal contractile response to KCl. In 6 out of 7 strips L-NOARG also enhanced or unmasked the rebound contraction.

Neither L-NMMA (1 mM) nor L-NOARG (30 μM) reduced isoprenaline-(10 μM) induced relaxation ($n = 4$ for each compound). Likewise, relaxation produced by a submaximal concentration of isoprenaline (0.3 μM, about 30% of maximal response) was not reduced by the NO synthase inhibitors ($n = 2$ for each drug).

Discussion

The present findings indicate that L-NMMA and L-NOARG, two inhibitors of NO synthase, exert a partial inhibitory effect on the relaxation of the circular muscle of the human ileum evoked by electrical field stimulation of NANC nerves. The effect of L-NMMA and L-NOARG was reversed by L-Arg, in agreement with previous studies in which NO synthase inhibitors have been shown to block electrically-evoked, nerve mediated NANC relaxation in the mammalian gut (see Introduction for references). Blockade of NANC relaxation by

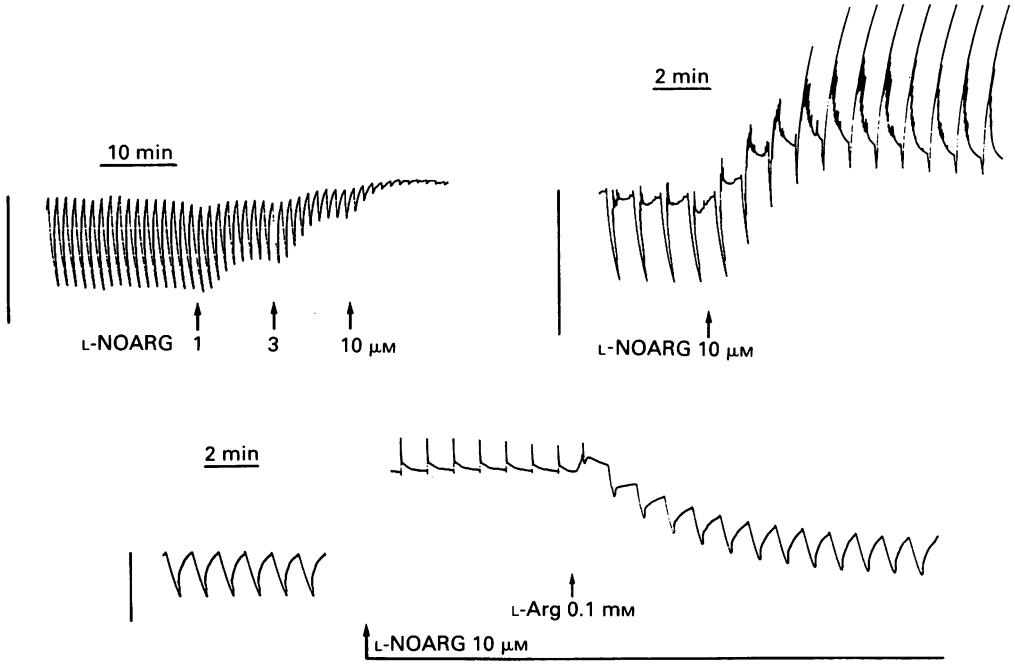

Figure 3 Original tracings showing the effect of N^G-nitro L-arginine (L-NOARG) on evoked non-adrenergic non-cholinergic relaxations (electrical field stimulation at 2 Hz) of the circular muscle of the human ileum and in the lower panel, reversal of L-NOARG inhibition by L-arginine (L-Arg). Vertical bars indicate maximal relaxation to isoprenaline.

L-NMMA in the human ileum was incomplete, averaging 35–40% at frequencies of 2–10 Hz. The inhibitory effect of L-NOARG was much more intense, averaging 65% but complete inhibition was also observed in individual strips. The greater potency and effectiveness of L-NOARG as compared to L-NMMA in the human ileum is consistent with the hypothesis of NO involvement in NANC relaxations, since previous studies have indicated a greater activity of L-NOARG as compared to L-NMMA in inhibiting NO generation (Moore *et al.*, 1990).

At present we have no conclusive explanation for the partial inhibitory effect of L-NOARG in the human ileum. The most likely possibility is that multiple inhibitory transmitters are involved in NANC relaxation of the human gut. ATP does not seem a likely candidate for NANC relaxation in the human ileum because it produces a contraction rather than relaxation (Maggi, unpublished observations). On the other hand, VIP produces a tetrodotoxin-resistant, slowly developing relaxation (Maggi *et al.*, 1990) and could still be considered as a possible mediator of NANC relaxation. The participation of both VIP and NO in nerve-mediated NANC relaxations of the rat isolated stomach has been proposed recently (Li & Rand, 1990). Although further studies are needed to elucidate fully the nature of transmitters responsible for NANC relaxation of the human gut, the present findings provide circumstantial evidence that NO, generated during evoked nerve activity, may be involved.

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Effects of zaprinast and rolipram on platelet aggregation and arrhythmias following myocardial ischaemia and reperfusion in anaesthetized rabbits

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1 This study was designed to compare the effects of two selective inhibitors of certain phosphodiesterase (PDE) isoenzymes on arrhythmias induced by coronary artery occlusion and reperfusion. The drugs used were zaprinast which inhibits guanosine 3':5'-cyclic monophosphate (cyclic GMP)-specific PDE (PDE V) and rolipram which inhibits cyclic GMP-insensitive, adenosine 3':5'-cyclic monophosphate (cyclic AMP)-specific PDE (PDE IV).

2 Pretreatment of anaesthetized rabbits with zaprinast ($300\,\mu g\,kg^{-1}$ plus $30\,\mu g\,kg^{-1}\,min^{-1}$) had no significant effect on ischaemia- or reperfusion-induced ST-segment changes, or arrhythmias. In contrast, rolipram ($30\,\mu g\,kg^{-1}$ plus $3\,\mu g\,kg^{-1}\,min^{-1}$) and ($100\,\mu g\,kg^{-1}$ plus $10\,\mu g\,kg^{-1}\,min^{-1}$) increased the severity of arrhythmias. With the higher dose of rolipram, ST-segment changes were increased in magnitude and mortality due to ventricular fibrillation during ischaemia or reperfusion was increased to 80% compared with 30% in controls ($n = 10$ per group).

3 Zaprinast caused small but significant increases in heart rate and arterial blood pressure whereas rolipram decreased diastolic arterial pressure, increased left ventricular (LV) dP/dt_{max} and substantially increased heart rate.

4 At the end of each experiment platelet aggregation was measured *ex vivo*. Pretreatment of rabbits with either dose of rolipram had no significant effect on platelet aggregation induced by adenosine diphosphate (ADP), collagen, arachidonic acid or thrombin or on isoprenaline- or prostacyclin-induced inhibition of aggregation. Aggregatory responses to ADP and collagen were increased in platelets obtained from rabbits which had received zaprinast.

5 These results indicate that in the dose used here, the PDE V inhibitor zaprinast had no significant effect on arrhythmias. The effects of the PDE IV inhibitor rolipram on haemodynamics, combined with its lack of antiplatelet activity, may have contributed to the exacerbation of arrhythmias observed during myocardial ischaemia and reperfusion.

Keywords: Arrhythmias; myocardial ischaemia; reperfusion; zaprinast; M&B 22,948; rolipram; phosphodiesterase inhibitors; platelet aggregation

Introduction

We have demonstrated previously that the non-selective phosphodiesterase (PDE) inhibitor isobutylmethylxanthine (IBMX) had antiarrhythmic activity in anaesthetized rabbits subject to acute coronary artery occlusion, whereas the selective PDE III inhibitor, milrinone had no significant antiarrhythmic effects (Holbrook & Coker, 1989). Although the mechanism(s) responsible for the antiarrhythmic action of IBMX could not be determined from this previous study, alterations in platelet aggregation did not seem to be directly relevant since milrinone had greater anti-platelet activity than IBMX. One possible explanation for the antiarrhythmic effect of IBMX was that it may be related to the ability of this compound to increase guanosine 3':5'-cyclic monophosphate (cyclic GMP) rather than adenosine 3':5'-cyclic monophosphate (cyclic AMP).

In the last decade several different PDE isoenzymes have been identified and it has been suggested recently that there may be at least five distinct, but related, gene families coding for cyclic nucleotide PDEs (see Beavo & Reifsnyder, 1990, for descriptions and nomenclature). IBMX inhibits all PDEs whereas milrinone is selective for the cyclic GMP-inhibited, cyclic AMP-specific PDE (PDE III). Selective increases in cyclic AMP alone, can also be achieved by inhibition of the cyclic GMP-insensitive, cyclic AMP-specific PDE (PDE IV) with a drug such as rolipram. Conversely, zaprinast will increase cyclic GMP by inhibiting the cyclic GMP-specific PDE (PDE V, previously termed PDE I, e.g. see Weishaar, 1987).

Both cyclic AMP and cyclic GMP can mediate vasodilator responses and impair platelet aggregation (Packham & Mustard, 1980; Waldman & Murad, 1987; Lincoln, 1989). During acute myocardial ischaemia, alterations in vascular tone or platelet aggregation in the coronary circulation may be important determinants of the severity of subsequent arrhythmias. Thus the aim of the present study was to extend our knowledge of the consequences of selective PDE inhibition by examining the effects of rolipram and zaprinast in our anaesthetized rabbit model of acute myocardial ischaemia and reperfusion.

Methods

Animal preparation

Experiments were performed in male New Zealand White rabbits (2.1 to 3.2 kg) purchased from Hylyne, Cheshire. Anaesthesia was induced by i.m. injection of diazepam $2.5\,mg\,kg^{-1}$ followed by Hypnorm $0.4\,ml\,kg^{-1}$. Rabbits were then prepared for coronary artery occlusion as described previously (Coker, 1989). Briefly, a Lead II ECG was recorded along with arterial blood pressure, left ventricular pressure and its first derivative with time. Drugs were administered into the vena cava via a right femoral venous catheter. After cannulation of the trachea, sodium pentobarbitone was administered i.v. (24 to $48\,mg\,kg^{-1}$) to maintain anaesthesia.

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A left thoracotomy was performed at the fourth intercostal space and the rabbits were ventilated with room air at 38 strokes per min, 12 to 18 ml per stroke, with a positive end expiratory pressure of 1 to 2 cmH_2O. Arterial blood gases and pH were measured (Corning 158 Analyzer) and stroke volume was adjusted to maintain P_{CO_2} within normal limits. The pericardium was incised and a fine silk ligature was placed around the major anterolateral branch of the left circumflex coronary artery.

Experimental protocol

A stabilization period of at least 15 min was allowed after the completion of the surgical procedures. A drug or vehicle was then administered i.v. as an initial bolus dose followed immediately by a continuous infusion. Zaprinast was given at a dose of 300 $\mu g\,kg^{-1}$ plus 30 $\mu g\,kg^{-1}\,min^{-1}$ whereas two doses of rolipram were studied; 30 $\mu g\,kg^{-1}$ plus 3 $\mu g\,kg^{-1}\,min^{-1}$ and 100 $\mu g\,kg^{-1}$ plus 10 $\mu g\,kg^{-1}\,min^{-1}$. Control rabbits received equivalent volumes of the vehicle (25% v/v polyethylene glycol 300 in water). There were 10 rabbits in each group. The coronary artery was occluded 10 min after commencing drug administration and myocardial ischaemia was maintained for 20 min, after which time the ligature around the coronary artery was released to allow reperfusion. At the end of each experiment, i.e. after 10 min of reperfusion or after 3 min of continuous ventricular fibrillation (if this occurred), blood was removed from the right ventricle via a 21G needle to permit the study of platelet aggregation *ex vivo*. The heart was then removed, the aorta cannulated and after retying the ligature around the coronary artery, a 1% w/v solution of Coomassie Blue was injected retrogradely into the aorta to stain the non-ischaemic myocardium. After dissecting the unstained tissue from the rest of the ventricles, both ischaemic and normal regions were weighed. The 'area at risk' was calculated as a percentage of the total ventricular mass. Changes in the ST-segment of the ECG and ischaemia- and reperfusion-induced arrhythmias were analyzed as described previously (Coker, 1989).

Exclusion criteria

Animals were excluded from further study or final analysis if any of the following occurred: (a) arrhythmias prior to coronary artery occlusion; (b) ST-segment changes prior to coronary artery occlusion; (c) area at risk less than 30% of total ventricular mass; (d) area at risk greater than 60% of total ventricular mass.

Platelet aggregation

Blood samples were placed in plastic tubes containing 3.8% w/v trisodium citrate solution (1 ml to 9 ml blood) and centrifuged at 220 *g* for 10 min. The supernatant, platelet rich plasma (PRP) was removed and the remnants recentrifuged at 2000 *g* to give platelet poor plasma (PPP). The number of platelets in the PRP was counted and then the PRP was diluted with PPP to give a final platelet count of 2.5 to 3.0 $\times 10^5\,\mu l^{-1}$. Aliquots of 100 μl of PRP were placed in cuvettes in a Payton dual channel aggregometer and stirred at 900 r.p.m. at 37°C. After an equilibration period of 3 min, aggregating agents were added and platelet aggregation was measured as the change in light transmission with the aggregometer set so that light transmission was 0% with PRP and 100% with PPP. In experiments where inhibition of platelet aggregation was studied, the inhibitory agent (isoprenaline or prostacyclin) was added 1 min before the aggregating agent (ADP).

Drugs

Zaprinast (M&B 22,948) and rolipram were gifts from Rhone-Poulenc, Dagenham and Schering AG, Berlin respectively. Due to its limited solubility, rolipram was dissolved in 25% v/v polyethylene glycol 300 in water with the aid of sonication. This vehicle was also used for zaprinast and was given in appropriate volumes to the control rabbits. Adenosine diphosphate (ADP), arachidonic acid, collagen, isoprenaline, prostacyclin and thrombin were purchased from Sigma, Poole; trisodium citrate from BDH, Poole; diazepam injection from the Royal Liverpool Hospital Pharmacy; Hypnorm (which contains 0.315 mg fentanyl citrate and 10 mg fluanisone per ml) from Janssen, Wantage and sodium pentobarbitone (Sagatal) from Rhone-Poulenc, Dagenham.

Statistics

Where appropriate, values have been expressed as the mean $\pm$ s.e.mean of n experiments. Comparisons between groups were made with an unpaired t test and within groups with a paired t test. The incidence of events was compared by Fisher's exact test.

Results

In total, 44 rabbits were used in this study. Of these, 4 were excluded; 2 which had ventricular premature beats (VPBs) prior to coronary artery occlusion and 2 in which the area of ventricle at risk of ischaemia was below the acceptable minimum (<30%). This left 10 rabbits in each group, all of which had similar sized areas at risk of ischaemia. The values were 42 $\pm$ 3% of total ventricular mass in controls; 39 $\pm$ 4% in the zaprinist group; 38 $\pm$ 3% in the low dose rolipram group and 41 $\pm$ 3% in the high dose rolipram group.

Arrhythmias and ST-segment changes

During the 20 min period of myocardial ischaemia VPBs were observed in all rabbits except one in the control group, whereas following reperfusion, ectopic activity occurred in all rabbits. Pretreatment with zaprinast did not significantly alter the incidence of ventricular tachycardia, ventricular fibrillation or the mortality resulting from acute myocardial ischaemia or reperfusion (Figure 1). In contrast the severity of arrhythmias was increased in the rabbits which received rolipram, resulting in an increase in mortality (due to terminal ventricular fibrillation) following the combined insult of ischaemia plus reperfusion (Figure 1).

Normally, in this model, ischaemia-induced ventricular tachycardia is a rare event and in this particular study none of the rabbits in the control group or the zaprinast-treated group had ventricular tachycardia during myocardial ischaemia. In the group of rabbits which received the lower dose of rolipram, however, 70% had ventricular tachycardia during the ischaemic period ($P = 0.003$ compared with control, Fisher's exact test). Although only 20% of the rabbits pretreated with the higher dose of rolipram had ventricular tachycardia, this was because most of the animals in this group went straight into ventricular fibrillation without a preceding episode of ventricular tachycardia.

Ventricular fibrillation occurred during ischaemia in 5 out of the 10 control rabbits but in 2 of these animals it was self-terminating (see Figure 1). In all other rabbits in which ventricular fibrillation occurred it was sustained, resulting in death. Most ventricular fibrillation occurred during ischaemia but 3 of the remaining rolipram-treated rabbits fibrillated following reperfusion (Figure 1). As well as increasing the severity of arrhythmias, ventricular fibrillation also appeared to occur earlier in the rabbits which received rolipram. In controls, ventricular fibrillation is most frequently observed between 10 and 15 min after coronary artery occlusion. In 1 rabbit which received the higher dose of rolipram, ventricular fibrillation occurred during the 3rd min of myocardial ischaemia and 4 rabbits had fibrillated by 10 min post-occlusion (see Figure 2, upper panel).

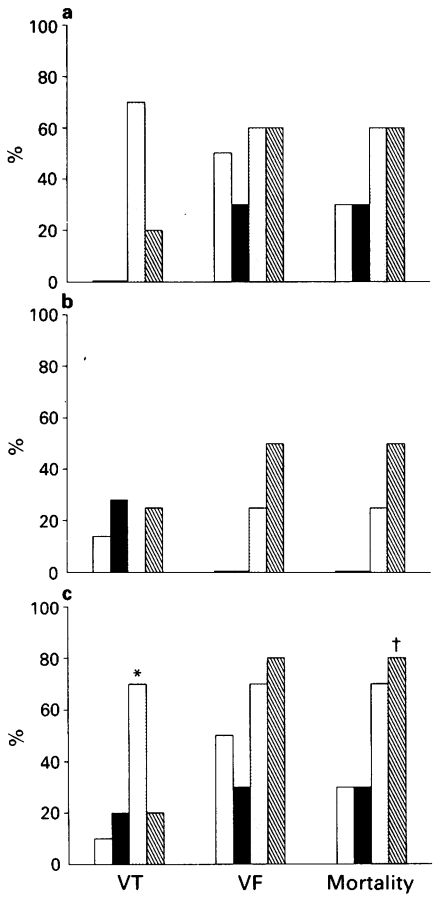

Figure 1 The incidence of ventricular tachycardia (VT), ventricular fibrillation (VF) and the mortality (a) during myocardial ischaemia, (b) following reperfusion in survivors and (c) the total values after ischaemia plus reperfusion, in controls (open columns) and in rabbits pretreated with zaprinast (solid columns), low dose rolipram (stippled columns) or high dose rolipram (hatched columns), $n = 10$ per group. * $P = 0.019$; † $P = 0.070$, compared with corresponding value in controls, Fisher's exact test.

Significantly greater changes in the ST-segment of the ECG were observed in the rabbits which received the higher dose of rolipram. Although the mean values for ST-segment change during ischaemia (Figure 2) also appear to be greater in the zaprinast-pretreated rabbits the variation in values within this group was larger, and thus the differences from the control group were not statistically significant. To avoid the risk of generating false positives by performing repetitive t tests, comparisons were made only at two time points; 7 min post-occlusion, which is before arrhythmias start in most controls, and at the end of the ischaemic period (i.e. 20 min post-occlusion).

Haemodynamics and blood gases

Within 1 min of starting drug administration, significant haemodynamic changes were observed. With zaprinast, heart rate and arterial blood pressure were increased and remained elevated after 10 min of drug infusion (Table 1). The lower dose of rolipram caused sustained increases in heart rate and left ventricular dP/dt_{max} but only a transient increase in systolic blood pressure. After 10 min of infusion of this lower dose of rolipram a reduction in diastolic blood pressure was evident. The same pattern of responses was observed with the higher dose of rolipram although the magnitude of some changes was greater (Table 1).

In control rabbits coronary artery occlusion caused immediate reductions in heart rate, systolic blood pressure and left ventricular dP/dt_{max} which gradually reversed with time. By the end of the ischaemic period the only significant haemodynamic change was an increase in left ventricular end-diastolic pressure (Table 1). Similar ischaemia-induced alterations in haemodynamics were observed in the drug-treated rabbits, with the exception that no reduction in heart rate was seen in either group that had received rolipram.

Arterial blood gases and pH were measured before drug administration and again just before coronary artery occlusion, i.e. after 10 min of drug administration. The values are detailed in Table 2. There were no differences between the baseline values in any of the groups and none of the drugs altered arterial Po_2, Pco_2 or pH.

Platelet aggregation

Platelets obtained from all of the rabbits aggregated in response to ADP and arachidonic acid. The magnitude of the responses to arachidonic acid (10^{-3} M) was similar in all the groups: 51 ± 3% in controls, 52 ± 3% zaprinast, 50 ± 3% low dose rolipram and 53 ± 3% high dose rolipram. In contrast, although ADP produced concentration-dependent platelet aggregation in all the groups, some of the responses were greater in platelets from rabbits that had received zaprinast (Figure 3). The effects of ADP on the aggregation of platelets obtained from rabbits that had received rolipram

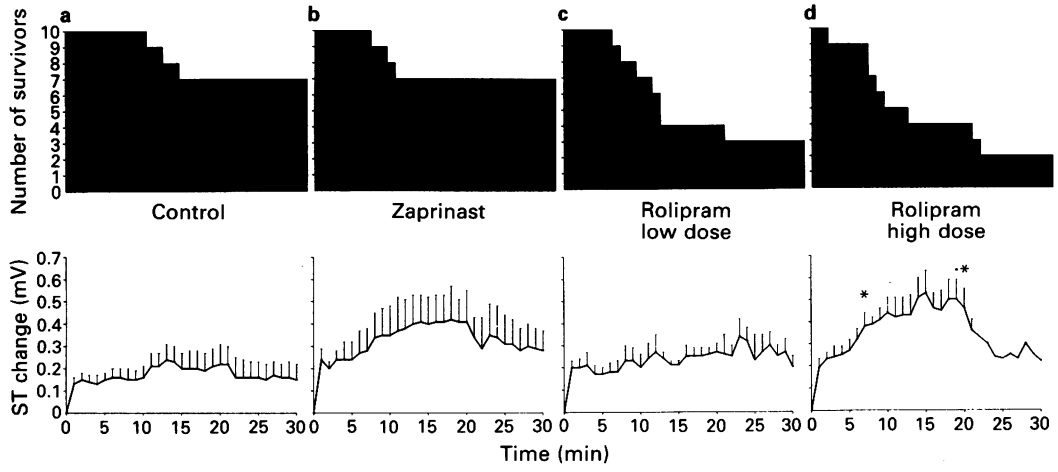

Figure 2 The number of survivors and the ST-segment change (mean with vertical bars indicating the s.e.mean) following coronary artery occlusion at time 0 and reperfusion at 20 min in controls (a) and in rabbits pretreated with zaprinast (b), low dose rolipram (c) or high dose rolipram (d).
* $P < 0.05$ compared with corresponding time point in controls, independent t test.

Table 1 The effects of drug (or vehicle) administration at −10 min, coronary artery occlusion at time 0 and reperfusion at 20 min on heart rate (HR), systolic and diastolic blood pressures (SBP and DBP), left ventricular end-diastolic pressure (LVEDP) and left ventricular (LV) dP/dt_{max}

Time (min)	n	*HR* (beats min^{-1})	*SBP* (mmHg)	*DBP* (mmHg)	*LVEDP* (mmHg)	*LV*dP/dt$_{max}$ (mmHg s^{-1})
Control						
−11	10	293 ± 10	76 ± 4	45 ± 3	3.5 ± 0.5	3640 ± 130
−9	10	294 ± 10	80 ± 3	48 ± 2		3690 ± 130
−1	10	292 ± 10	80 ± 3	47 ± 3	3.2 ± 0.4	3720 ± 120
1	10	287 ± 8†	72 ± 4††	45 ± 4		3330 ± 130†††
19	7	279 ± 13	77 ± 2	48 ± 2	8.4 ± 2.0†	3290 ± 130
30	7	288 ± 13	79 ± 4	50 ± 3	3.3 ± 0.5	3440 ± 190
Zaprinast (300 μg kg^{-1} plus 30 μg kg^{-1} min^{-1})						
−11	10	291 ± 10	84 ± 3	50 ± 3	3.8 ± 0.4	3680 ± 200
−9	10	299 ± 10*	99 ± 3***	61 ± 4**		3940 ± 290
−1	10	301 ± 9*	98 ± 3***	58 ± 5	3.8 ± 0.7	3970 ± 270
1	10	291 ± 10††	88 ± 6†††	51 ± 9†		3430 ± 240††
19	7	281 ± 12	89 ± 5	47 ± 4	8.4 ± 2.0†	3290 ± 250
30	7	289 ± 13	92 ± 5	48 ± 4	5.3 ± 1.4	3500 ± 170
Rolipram (30 μg kg^{-1} plus 3 μg kg^{-1} min^{-1})						
−11	10	283 ± 10	83 ± 3	52 ± 5	3.1 ± 0.4	3500 ± 70
−9	10	313 ± 10***	89 ± 3**	53 ± 4		3690 ± 90**
−1	10	317 ± 8***	81 ± 3	46 ± 4*	3.6 ± 0.7	3690 ± 70***
1	10	312 ± 9	73 ± 5††	40 ± 5		3110 ± 170††
19	4	308 ± 16	79 ± 6	51 ± 9	6.0 ± 1.1†	3440 ± 120
30	3	317 ± 17	80 ± 10	54 ± 10	2.8 ± 1.5	3290 ± 110
Rolipram (100 μg kg^{-1} plus 10 μg kg^{-1} min^{-1})						
−11	10	273 ± 8	78 ± 4	45 ± 4	2.7 ± 0.3	3360 ± 100
−9	10	310 ± 10***	84 ± 4***	46 ± 4		4030 ± 180***
−1	10	316 ± 12***	77 ± 5	38 ± 2*	3.3 ± 0.6	3840 ± 130***
1	10	316 ± 13	68 ± 4†	40 ± 4		3090 ± 230††
19	4	289 ± 20	71 ± 3	37 ± 5	5.6 ± 0.8††	3410 ± 80
30	2	328 ± 15	68 ± 8	41 ± 6	6.8 ± 3.3	3190 ± 60

Each value is the mean ± s.e.mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with pre-drug (−11 min) value; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ compared with pre-occlusion (−1 min) value, paired t test.

Table 2 Arterial blood gases and pH measured before drug administration and after 10 min of drug infusion

		pH (units)	Pco_2 (mmHg)	Po_2 (mmHg)
Control	Pre-drug	7.45 ± 0.02	36.8 ± 0.7	94.6 ± 3.2
	Post-drug	7.44 ± 0.01	37.0 ± 0.8	95.0 ± 3.8
Zaprinast	Pre-drug	7.44 ± 0.03	37.2 ± 0.3	98.8 ± 4.6
	Post-drug	7.45 ± 0.02	36.3 ± 0.4	94.7 ± 3.7
Rolipram low dose	Pre-drug	7.43 ± 0.03	36.1 ± 0.1	89.6 ± 3.0
	Post-drug	7.44 ± 0.03	36.8 ± 0.3	92.1 ± 2.9
Rolipram high dose	Pre-drug	7.44 ± 0.02	37.0 ± 0.1	99.9 ± 2.7
	Post-drug	7.44 ± 0.02	36.8 ± 0.3	100.5 ± 3.1

Each value is the mean ± s.e.mean. $n = 10$ per group.

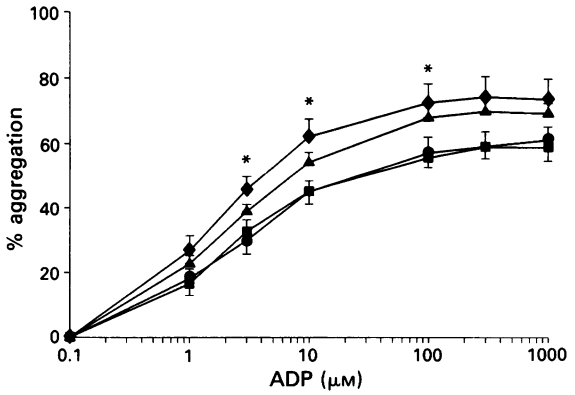

Figure 3 ADP-induced aggregation of platelets from control rabbits (●) and from rabbits that had received zaprinast (◆), low dose rolipram (▲) or high dose rolipram (■). Each value is the mean with vertical bars indicating the s.e.mean, $n = 10$ per group. * $P < 0.05$ compared with corresponding value in controls, independent t test.

were not significantly different from control. Thrombin (2 units ml^{-1}) caused the aggregation of platelets from 70 to 90% of the rabbits in each group. Although there were no significant differences in the number of rabbits in each group whose platelets responded to collagen, the magnitude of the response was significantly enhanced in platelets from rabbits pretreated with zaprinast (Table 3).

The addition of isoprenaline (10^{-8} to 10^{-5} M) to platelets prior to ADP (10^{-5} M) caused concentration-dependent inhibition of the ADP-induced platelet aggregation (Figure 4). At higher concentrations of isoprenaline (10^{-4} and 10^{-3} M) this effect started to reverse, presumably due to additional stimulation of α_2-adrenoceptors by isoprenaline. In platelets from rabbits that had received either zaprinast or rolipram, the effects of isoprenaline on ADP-induced aggregation were not different from those in controls (Figure 4). Prostacyclin also caused concentration-dependent inhibition of ADP-induced platelet aggregation, with no differences observed between the responses in platelets from control rabbits or those that had received either phosphodiesterase inhibitor (Figure 5).

Table 3 The number of rabbits whose platelets responded and the magnitude of the response to collagen ($0.2\,mg\,ml^{-1}$)

	Number responding	*% aggregation*
Control	6/10	54 ± 8
Zaprinast	8/10	86 ± 6*
Rolipram low dose	7/10	71 ± 7
Rolipram high dose	8/10	65 ± 4

Values are the mean ± s.e.mean. * $P < 0.01$ compared with control, unpaired t test.

Discussion

The results of this study indicate that zaprinast (in the dose used here, $300\,\mu g\,kg^{-1}$ plus $30\,\mu g\,kg^{-1}\,min^{-1}$) had no significant effects on ischaemia- or reperfusion-induced arrhythmias. In contrast, rolipram exacerbated arrhythmias during acute myocardial ischaemia and reperfusion, to the extent that mortality due to sustained ventricular fibrillation was significantly increased in the group that received the higher dose of rolipram. This latter result suggests that increasing cyclic AMP, by inhibition of PDE IV, in the setting of acute myocardial ischaemia has detrimental effects.

In some respects this is hardly surprising since a large amount of evidence has accumulated which implicates local increases in myocardial cyclic AMP in the genesis of ischaemia-induced arrhythmias (see Podzuweit, 1982). Both clinical and experimental studies have suggested that PDE III inhibitors such as amrinone and milrinone can have arrhythmogenic effects during myocardial ischaemia or reperfusion (Collucci *et al.*, 1986; Lukas & Ferrier, 1988; Lynch *et al.*, 1989). Although we did not see any significant arrhythmogenic activity with milrinone in our previous study in rabbits (Holbrook & Coker, 1989) it is possible that a higher dose may have exacerbated arrhythmias.

The doses of rolipram used in the present study were chosen on the basis of its reported relative potency as an inhibitor of certain PDE isoenzymes and from preliminary experiments on its effects on heart rate, arterial blood pressure and $LVdP/dt_{max}$ in anaesthetized rabbits in our laboratory. Although rolipram is slightly less potent as an inhibitor of PDE IV than milrinone is as an inhibitor of PDE III (Beavo, 1988), we decided to use rolipram in the same doses that we had used milrinone previously (Holbrook & Coker, 1989). A major reason for this decision was that we wanted to avoid causing large reductions in arterial pressure with rolipram because we had evidence that the rabbits which received milrinone and subsequently fibrillated during myocardial ischaemia were those in which milrinone caused greater decreases in blood pressure (see Holbrook & Coker, 1989). The reduction in diastolic blood pressure observed 10 min after starting administration of rolipram was almost identical to that seen previously with milrinone but in the present study there were no differences in pressures between the rolipram-treated rabbits that died and those that survived. Thus it is unlikely that the detrimental effects of rolipram on arrhythmias were due to reductions in diastolic blood pressure.

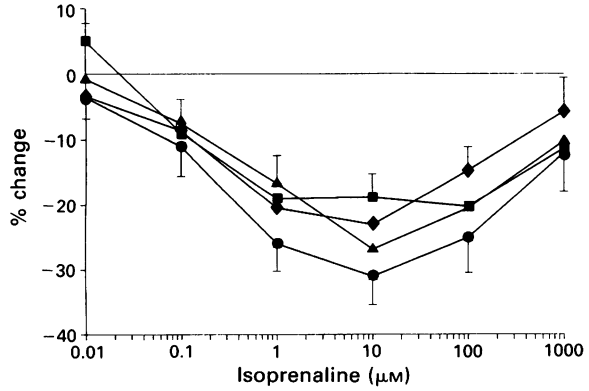

Figure 4 The effect of isoprenaline on 10^{-5} M ADP-induced aggregation of platelets from control rabbits (●) and from rabbits that had received zaprinast (◆), low dose rolipram (▲) or high dose rolipram (■). Each value is the mean with vertical bars indicating the s.e.mean, $n = 10$ per group. Some error bars have been omitted for clarity.

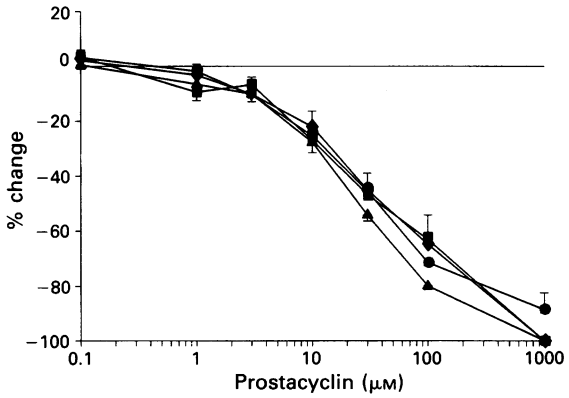

Figure 5 The effect of prostacyclin on 10^{-5} M ADP-induced aggregation of platelets from control rabbits (●) and from those that had received zaprinast (◆), low dose rolipram (▲) or high dose rolipram (■). Each value is the mean with vertical bars indicating the s.e.mean, $n = 10$ per group. Some error bars have been omitted for clarity.

In the present study, the haemodynamic effects of rolipram were different in some ways from those of milrinone, since systolic blood pressure was maintained and $LVdP/dt_{max}$ was increased by rolipram. Although PDE IV has been reported to be present in ventricular tissue in the rabbit (Kithas *et al.*, 1988; Shahid & Nicholson, 1990) and other species (Weishaar *et al.*, 1987) it has been observed that rolipram alone appears to be devoid of inotropic activity *in vitro* (Weishaar *et al.*, 1987; Muller *et al.*, 1990; Shahid & Nicholson, 1990). However, when cyclic AMP is increased either by stimulation of adenylate cyclase or by inhibition of PDE III, rolipram can have positive inotropic actions (Muller *et al.*, 1990; Shahid & Nicholson, 1990). Thus the increased myocardial contractility observed here *in vivo* with rolipram suggests that cyclic AMP may already have been elevated. This could simply be a reflection of the level of sympathetic tone in these anaesthetized rabbits or it may be related to the ability of rolipram to increase noradrenaline turnover (Wachtel, 1983). Elevated catecholamine concentrations would increase cyclic AMP further, thus providing an additional mechanism for the exacerbation of ischaemia-induced arrhythmias by rolipram.

The increases in heart rate resulting from the administration of rolipram may also have contributed to the greater incidence of ventricular fibrillation. Positive correlations between heart rate and the occurrence of ischaemia-induced and reperfusion-induced ventricular fibrillation have been reported (Coker & Parratt, 1985; Bolli *et al.*, 1986). In the control rabbits (and those which received zaprinast), 1 min after coronary artery occlusion heart rate was significantly reduced suggesting increased vagal activity. It has been proposed that the balance between sympathetic and parasympathetic tone may be an important factor in arrhythmogenesis during ischaemia (Zaza & Schwartz, 1985), with increased vagal activity being associated with reductions in ventricular fibrillation (Collins & Billman, 1989; Billman, 1990). No ischaemia-induced reductions in heart rate were observed in the rabbits that had received rolipram which again suggests that rolipram may have elevated sympathetic tone.

It is possible that increased oxygen demand (due to increased heart rate and myocardial contractility) may have contributed to the increased severity of arrhythmias observed

in rabbits which received rolipram. However, calculation of the rate-pressure product, an estimate of oxygen demand, revealed that zaprinast increased this parameter as much as either dose of rolipram (see Table 1 for mean rates and pressures). Since zaprinast did not exacerbate arrhythmias it seems unlikely that increased oxygen demand could account for the effect of rolipram.

Another possible explanation for the difference between the effects of rolipram and milrinone on arrhythmias involves platelets. In our previous study, milrinone did not alter arrhythmias but had significant antiplatelet activity (Holbrook & Coker, 1989). Under identical conditions, pretreatment of rabbits with rolipram had no effect on platelet aggregation measured *ex vivo*. The lack of activity of rolipram is probably due to the absence of PDE IV in platelets (Simpson *et al.*, 1988; Shahid *et al.*, 1990). We have been unable to demonstrate the presence of a rolipram-sensitive, cyclic GMP-insensitive, cyclic AMP-specific PDE (PDE IV) in rabbit platelets (Holbrook *et al.*, 1990). Thus rolipram, unlike milrinone, may exacerbate ischaemia-induced arrhythmias because the arrhythmogenic actions of increasing myocardial cyclic AMP cannot be offset by the antiarrhythmic consequences of reducing platelet aggregation (via increased platelet cyclic AMP).

The enhancement of ADP- and collagen-induced aggregation of platelets obtained from the rabbits that had received zaprinast was surprising since increases in cyclic GMP (e.g. induced by nitrovasodilators or endothelium-derived relaxing factor (EDRF)) have been associated with reduced platelet aggregation (Hogan *et al.*, 1988; Lidbury *et al.*, 1989; Willis *et al.*, 1989). Similarly the haemodynamic effects of zaprinast were unexpected. Zaprinast has been shown to decrease arterial blood pressure in anaesthetized and conscious rats (Banerjee *et al.*, 1989; McMahon *et al.*, 1989; Dundore *et al.*, 1990) albeit at much higher doses than that used here. The dose of zaprinast was chosen on the basis of its reported activity against PDE V relative to its ability to inhibit other PDE isoenzymes (Beavo, 1988). We decided not to use a higher dose of zaprinast since we wanted to use a dose which would only increase cyclic GMP by inhibition of PDE V. It is possible, however, that the dose used ($300\,\mu g\,kg^{-1}$ plus $30\,\mu g\,kg^{-1}\,min^{-1}$) was insufficient to increase cyclic GMP *in vivo*. The data from the rat studies quoted above, which have been published since we started our experiments, do suggest that *in vivo*, high doses of zaprinast are required to produce significant decreases in blood pressure. It is interesting to note, however, that Dundore *et al.* (1990) reported a small but significant increase in arterial pressure with $3\,mg\,kg^{-1}$ zaprinast, which was similar to the increase in blood pressure that we observed here in rabbits, but they did not suggest any explanation for this effect of zaprinast. It is possible that the small increases in heart rate, arterial blood pressure and platelet aggregation observed in the present study were due to increased sympathetic tone, but whether this is a direct or reflex effect of zaprinast remains to be determined. Alternatively, the apparently enhanced aggregation of platelets from the rabbits that received zaprinast may simply be due to variability between groups.

In summary, therefore, we cannot draw any firm conclusions about the actions of zaprinast since only one dose was examined and no significant effects on arrhythmias were observed. Rolipram, however, was devoid of anti-platelet activity, increased heart rate and $LVdP/dt_{max}$ and increased the severity of arrhythmias induced by acute myocardial ischaemia and reperfusion in anaesthetized rabbits.

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Characterization of diadenosine tetraphosphate (Ap_4A) binding sites in cultured chromaffin cells: evidence for a P_{2y} site

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1 Diadenosine tetraphosphate (Ap_4A) a dinucleotide, which is stored in secretory granules, presents two types of high affinity binding sites in chromaffin cells. A K_d value of $8 \pm 0.65 \times 10^{-11}$ M and B_{max} value of 5420 ± 450 sites per cell were obtained for the high affinity binding site. A K_d value of $5.6 \pm 0.53 \times 10^{-9}$ M and a B_{max} value close to 70,000 sites per cell were obtained for the second binding site with high affinity.

2 The diadenosine polyphosphates, Ap_3A, Ap_4A, Ap_5A and Ap_6A, displaced [^{3}H]-Ap_4A from the two binding sites, the K_i values being 1.0 nM, 0.013 nM, 0.013 nM and 0.013 nM for the very high affinity binding site and 0.5 μM, 0.13 μM, 0.062 μM and 0.75 μM for the second binding site.

3 The ATP analogues displaced [^{3}H]-Ap_4A with the potency order of the P_{2y} receptors, adenosine 5'-O-(2 thiodiphosphate) (ADP-β-S) > 5'-adenylyl imidodiphosphate (AMP-PNP) > α,β-methylene ATP (α,β-MeATP), in both binding sites. The K_i values were respectively 0.075 nM, 0.2 nM and 0.75 nM for the very high affinity binding site and 0.125 μM, 0.5 μM and 0.9 μM for the second binding site.

Keywords: Chromaffin cells; diadenosine polyphosphates; diadenosine tetraphosphate binding sites; ectophosphodiesterases, P_2-purinoceptors

Introduction

The presence of diadenosine polyphosphates, namely adenosine-(5')-triphospho-(5')-adenosine (Ap_3A), adenosine-(5')-tetraphospho-(5')-adenosine (Ap_4A), and adenosine-(5')-pentaphospho-(5')adenosine (Ap_5A) has been described in the secretory granules of neural and non-neural cells (Luthje & Ogilvie, 1983; Rodriguez del Castillo *et al.*, 1988). Considerable amounts of Ap_3A and Ap_4A are co-stored with 5-hydroxytryptamine and adenine nucleotides in the dense granules of human platelets (Flodgaard & Klenow, 1982; Luthje & Ogilvie, 1983). These compounds are released to the extracellular media during thrombin-induced aggregation. Ap_3A causes a gradual aggregation of platelets; in contrast, Ap_4A competitively inhibits ADP-induced platelet aggregation (Luthje & Ogilvie, 1984; Louie *et al.*, 1988). Both compounds also have vasoactive properties on peripheral arteries with intact or damaged endothelium (Busse *et al.*, 1988).

Chromaffin cells, which can be considered as the homologues of adrenergic neurones, store significant amounts of Ap_4A and Ap_5A. The diadenosine polyphosphates are co-stored in secretory granules with catecholamines and ATP (Rodriguez del Castillo *et al.*, 1988) and exocytotically released by the action of secretagogues (Pintor *et al.*, 1991). Recently reported are the effects of Ap_3A, Ap_4A and Ap_5A on catecholamine release, all of which increase the basal secretion but have an inhibitory action in nicotine-evoked release from isolated chromaffin cells (Castro *et al.*, 1990).

The extracellular receptors for adenine nucleotides (P_2) have been characterized by the pioneering work of Burnstock, who proposed the existence of two subtypes, P_{2x} and P_{2y} (Burnstock & Kennedy, 1985). This family of receptors is now increasing in number, due to the availability of specific ligands (Gordon, 1986; Burnstock, 1989; Cusack & Hourani, 1989). P_2-purinoceptors can be linked to several effector systems. The P_{2x} receptors appear to be coupled to calcium channels (Benham & Tsien, 1987). P_{2y} receptors seem to stimulate the inositol phospholipid metabolism (Forsberg *et al.*, 1987; Cooper *et al.*, 1989; Boyer *et al.*, 1990).

Concerning the diadenosine polyphosphate receptors, attempts have been made to characterize them. In sensory neurones, Ap_4A and Ap_5A are able to activate the same receptors as adenosine 5'-triphosphate (ATP), but to a lesser extent (Krishtal *et al.*, 1988). No single type of receptor seems to exist, and analogies with P_{2x}, P_{2y}, or even some different type of purinoceptor have been suggested (Busse *et al.*, 1988; Hoyle 1990). The presence of ectophosphodiesterases able to hydrolyze the Ap_xA with high affinity needs to be considered when studying the extracellular actions and binding sites of diadenosine polyphosphates (Goldman *et al.*, 1986; Miras-Portugal *et al.*, 1990; Rotllán *et al.*, 1991).

Since Ap_4A mediates an inhibitory action on induced catecholamine release in chromaffin cells, the aim of the present study was to determine its possible binding sites, as well to characterize the subtype of purinoceptor involved.

Methods

Isolation and culture of chromaffin cells

Chromaffin cells were isolated from bovine adrenal glands according to the method of Miras-Portugal *et al.* (1985). The cells were isolated by collagenase (EC 3.4.24.3) action and purified through a percoll gradient, carefully collected and washed with Ca^{2+}, Mg^{2+}-free Locke solution. Finally, cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal calf serum, 50 μM cytosine arabinofuranoside and 10 μM fluorodeoxyuridine. Cells were plated in 6-well Costar cluster dishes and incubated at 37°C in 5% CO_2 and 95% air at a density of 3×10^6 cells per well. Cells were employed for binding studies during the first five days of culture. Cellular viability was studied by trypan blue exclusion.

Ap_4A binding experiments and displacement studies

[^{3}H]-Ap_4A (6.3 Ci mmol^{-1}) binding assays were performed with 3×10^6 cultured chromaffin cells. The culture medium

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was aspirated and cells were washed twice with 1 ml Locke solution before the experiments were carried out. The binding assays were carried out in a volume of 1 ml Locke solution containing graded concentrations of [^{3}H]-Ap_4A ranging from 0.025 nM to 2.5 nM. The incubation period was 6 h at a temperature of 4°C to avoid the ectophosphodiesterase activity present in these cells (Miras-Portugal *et al.*, 1990). No hydrolysis of Ap_4A occurred in these conditions. Controls were studied by means of high performance liquid chromatography (h.p.l.c.), at the end of incubation period. Incubation was terminated by aspirating the fluid and washing twice with 1 ml cold Locke solution containing 10 μM of non-labelled Ap_4A, after which 1 ml of 10% trichloroacetic acid was added. The precipitated material was scraped out of the plastic dish, placed in a vial with 10 ml of scintillation liquid for aqueous samples, and counted in a Beckman LS 3.801. Non-specific binding was obtained by incubation of 10 μM non-labelled Ap_4A and subtracting the result from assays. This represented 5%–10% of the total.

The displacement studies with adenosine receptor ligands (R-phenylisopropyl adenosine (R-PIA) and N-ethyl-carboxamidoadenosine (NECA)), nucleotide receptor ligands (5'-adenylyl-imidodiphosphate (AMP-PNP), α,β-methylene ATP, (α,β-MeATP), adenosine 5'-O-(2-thiodiphosphate) (ADP-β-S)), Ap_4A analogues (Ap_3A, Ap_5A, and Ap_6A) were carried out as described above for cultured cells. The [H^3]-Ap_4A concentration employed in these assays was 2.5×10^{-10} M, because this value was three times the K_d value obtained (K_d = 0.08 nM). An exponential graded concentration ranging from 10^{-12} to 10^{-3} M was generally employed for every compound under study. Binding and displacement studies were analyzed by the computer programme LIGAND (Mundson & Robard, 1980). At the end of the incubation period the cellular integrity was measured by trypan blue exclusion and the lactate dehydrogenase was measured in the incubation media (Bergmeyer, 1974); one aliquot medium was removed for h.p.l.c. analysis.

The h.p.l.c. was carried out with a Waters 600 E pump with automated gradient controller, a variable volume injector U6K, and a 481-LC Spectrophotometer λ max (Waters). The detector responses were recorded, as were the integrated areas and retention times, with a 745 data module integrator (Waters).

Chromatography was performed with a C_{18} reverse phase column (C_{18} id. 3.9 mm, 22 cm long from Kontron) according to Rodriguez del Castillo *et al.* (1988) and adapted by Torres *et al.* (1990) for the ectonucleotidase activities present in chromaffin cells. The mobile phase was 10 mM potassium phosphate, 2 mM tetrabutylammonium (PIC A), a final pH of 7.5 and 15% (v/v) of acetonitrile. The retention times were 3 min for AMP, 4 min for ADP, 5.2 min for ATP and 9.4 min for Ap_4A, at a flow rate of 2 ml min^{-1}.

Materials

Nucleoside analogues and nucleotides, Ap_3A, Ap_4A and Ap_5A were from Boehringer (Mannheim, F.R.G.). Ap_6A was purchased from Sigma (St. Louis, U.S.A.). Phosphodiesterase from *Crotalus durissus* (EC 3.1.4.1), alkaline phosphatase (EC 3.1.3.1) and collagenase (EC 3.4.24.3) were from Pharmacia (Upsala, Sweden). Culture media and products were from Gibco (Glasgow, U.K.). [^{3}H]-Ap_4A was obtained from Amersham (United Kingdom).

Results

Measurements of [^3H]-diadenosine tetraphosphate binding to cultured chromaffin cells

The presence of an asymmetrical ectophosphodiesterase in chromaffin cells is a very important factor to consider in the study of Ap_4A binding sites (Miras-Portugal *et al.*, 1990; Rotllán *et al.*, 1991). To prevent the Ap_4A destruction, the binding studies were done at 4°C. As shown in Figure 1, no Ap_4A hydrolysis was observed after 8 h of incubation.

The time course of [^{3}H]-Ap_4A association with chromaffin cells is shown in Figure 2. The equilibrium was reached after 6 h incubation. The value obtained for the rate constant for the association, k_{+1} was 1.65 h^{-1} nM^{-1} (4°C). Controls for cellular integrity showed that, at the end of the experimental time, all the cells excluded the trypan blue and no release of lactate dehydrogenase activity was observed.

Once the assay conditions were established, the binding experiments were carried out, and the data obtained represented as a Scatchard plot (Figure 3). The results were analyzed by the computer programme LIGAND (Mundson & Robard, 1980) showing the presence of two binding sites. The first one presented a very high affinity as indicated by its K_d value of 0.080 ± 0.006 nM. The number of high affinity binding sites, estimated by extrapolation to the abscissa scale, gave a value of 27 fmol/3 × 10^6 cells which corresponds to 5420 ± 450 receptors per cell. The second binding site showed lower affinity; the K_d value obtained was 5.6 ± 0.53 nM, roughly two orders of magnitude higher. The bound maximum obtained for this second component was 345 ± 30 fmol/3 × 10^6 cells which corresponds to a value close to 70,000 binding sites per cell.

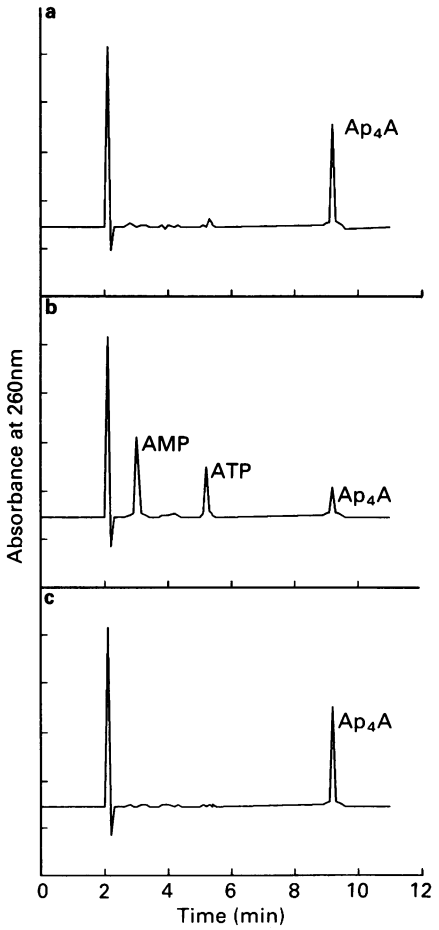

Figure 1 Ectophosphodiesterase activity in cultured chromaffin cells: (a) High performance liquid chromatography (h.p.l.c.) elution profile of 250 pmol diadenosine tetraphosphate (Ap_4A) standard in the elution condition as described in methods; (b) h.p.l.c. elution profile obtained with 3 × 10^6 cells incubated in the presence of 10 μM Ap_4A after 10 min incubation at 37°C. (c) The same as (b) but at 4°C and after 8 h incubation. Samples of 25 μl were injected. The first peak of the chromatogram corresponds with the solvent front and does not represent any adenosine compound.

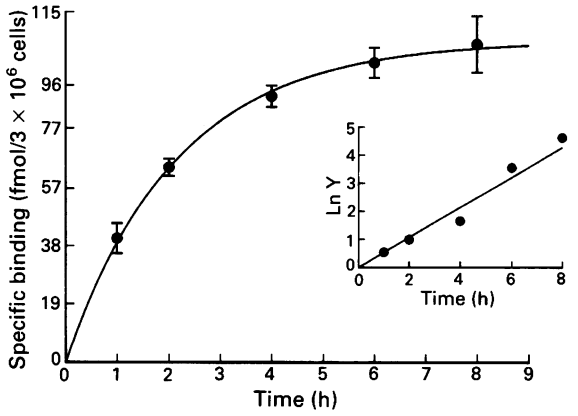

Figure 2 Determination of association rate for diadenosine tetraphosphate (Ap_4A) with cultured chromaffin cells at 4°C. The Ap_4A concentration was 0.250 nM. Controls of Ap_4A integrity were done for the maximal experimental time period (see Figure 1). In the insert, ln Y corresponds with $\text{Ln}\,Y = [B_e]/([B_e] - [B])$, where B_e is the bound of the equilibrium at this concentration and B is the bound at every experimental time. k_{+1} is deduced from the slope considering $k_{+1} = \text{slope}/([L]B_{max}/[B_e])$.

Displacement studies with diadenosine polyphosphates

Indirect binding assays of diadenosine polyphosphates, Ap_3A Ap_4A, Ap_5A and Ap_6A, were made by displacement studies of labelled [^{3}H]-Ap_4A. This compound was used at 0.25 nM, which is three times the high affinity K_d value (0.08 nM) in our experimental conditions.

In Figure 4 the displacement curves obtained for the diadenosine polyphosphates are shown. The percentage displacement with high affinity was about 10% of the total for each one.

The characteristic of Ap_4A, Ap_5A and Ap_6A displacement binding was very similar at the high affinity step (Table 1). Nevertheless, Ap_6A had a lower affinity for the second binding site. Ap_3A showed lower displacement capacity for both the high and the very high affinity binding sites. In Table 1 the K_i values for the two stepped shape of the curve are summarized.

Hill numbers (nH) confirmed the presence of more than one binding site. The nH values were very similar for all diadenosine polyphosphates studied, the mean value being 0.46 ± 0.18 (Table 1).

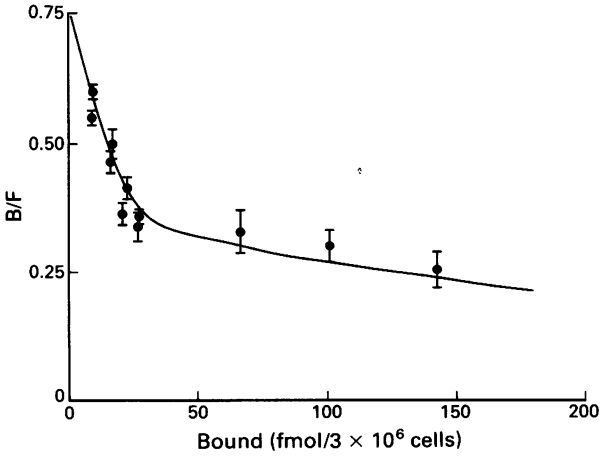

Figure 3 Scatchard analysis of equilibrium of [^{3}H]-diadenosine tetraphosphate ([^{3}H]Ap_4A) binding to chromaffin cells in culture. Binding was studied with 3 × 10^6 cells as described in methods. Results are means for six determinations in duplicate; s.d. shown by vertical bars. K_d and B_{max} values were obtained by the computer programme LIGAND (Mundson & Robard, 1980).

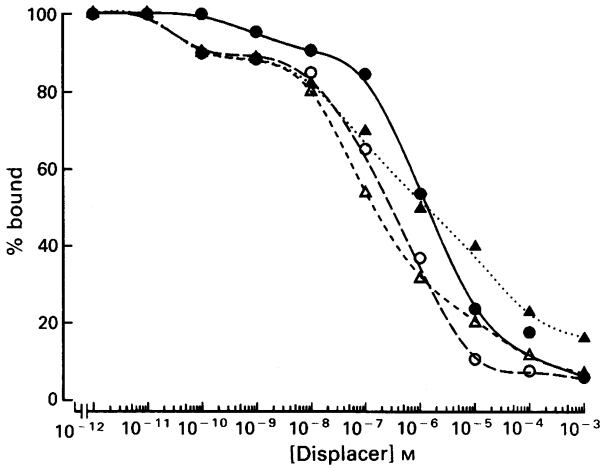

Figure 4 Inhibition of site-specific binding of [^{3}H]-diadenosine tetraphosphate ([^{3}H]-Ap_4A) to cultured chromaffin cells by Ap_xA compounds. Cells (3 × 10^6) were incubated with [^{3}H]-Ap_4A as described in methods in the presence of each, Ap_3A (●), Ap_4A (○), Ap_5A (△) or Ap_6A (▲). Results are plotted as a percentage of site-specific binding of [^{3}H]-Ap_4A in the absence of inhibitor. Results are means of three experiments in triplicate.

Binding displacement studies with P_1 and P_2 purinoceptor ligands

Compounds which have activity at P_2-purinoceptors were employed in displacement studies. The compounds used were ADP-β-S, AMP-PNP and α,β-MeATP, which are representative ligands of P_2-purinoceptors. The order is in the rank of potency for P_{2y} (Cooper *et al.*, 1989; Berrie *et al.*, 1989; Burnstock 1990; Cusack & Hourani, 1990). The displacement curves for these ligands are represented in Figure 5. As with the displacement of diadenosine polyphosphates, a two-stepped curve was obtained for each compound. The first step represented 10–12% of the total binding sites. ADP-β-S was the most potent displacer; however, it reached only one order of magnitude lower than Ap_4A (Table 1). AMP-PNP and α,β-Me-ATP, in this order of potency, were good displacers of

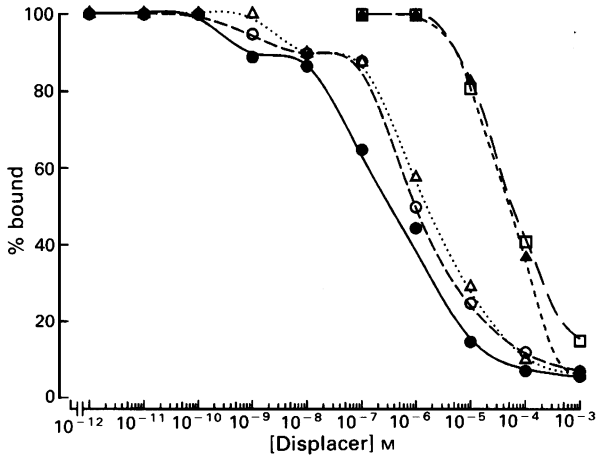

Figure 5 Displacement studies of diadenosine tetraphosphate (Ap_4A) binding by P_1 and P_2 receptor agonists. Cultured chromaffin cells (3 × 10^6) were incubated in the presence of P_1 receptor agonists phenylisopropyl adenosine (▲) and N-ethyl carboxamido adenosine (□) as described in methods. The P_2 agonists employed for the displacements were adenosine 5'-O-(2-thiodiphosphate) (●), 5'-adenylyl imidodiphosphate (○) and α,β methylene ATP (△). All the experiments represent the mean of three experiments in duplicate. The K_i values are summarized in Table 1.

Table 1 K_i values for the [^{3}H]-diadenosine tetraphosphate ([^{3}H]-Ap_4A) binding displacement by diadenosine polyphosphates, P_2 receptor agonists and P_1 agonists

Compound	(n)	*Very high affinity binding site* K_i (nM)	*High affinity binding site* K_i (μM)	*Hill number* nH
Ap_3A	(3)	1.000 ± 0.100	0.500 ± 0.050	0.49
Ap_4A	(3)	0.013 ± 0.002	0.130 ± 0.010	0.58
Ap_5A	(3)	0.013 ± 0.001	0.062 ± 0.006	0.38
Ap_6A	(3)	0.013 ± 0.002	0.750 ± 0.070	0.41
ADP-β-S	(3)	0.075 ± 0.006	0.130 ± 0.010	0.42
AMP-PNP	(3)	0.200 ± 0.015	0.500 ± 0.040	0.50
α,β-Me-ATP	(3)	0.750 ± 0.060	0.870 ± 0.070	0.60
NECA	(3)	—	15.000 ± 2.000	1.03
R-PIA	(3)	—	12.500 ± 1.500	1.02

The K_i values for the diadenosine polyphosphate analogues were obtained from the IC_{50} values of Figure 4. The values for P_1 and P_2 purinoceptor agonists were obtained from Figure 5. The programme ligand (Mundson & Robard, 1980) was employed. Values are the means ± s.d. of (*n*), number of individual experiments. The K_d value for the very high affinity binding site was 0.08 ± 0.0065 nM (6). The K_d value for the high affinity binding site was 5.6 ± 0.53 nM (6).
ADP-β-S: adenosine 5'-O-(2-thiodiphosphate); AMP-PNP: 5'-adenylyl imidodiphosphate; α,β Me-ATP: α,β methylene adenosine 5'-triphosphate; NECA: N-ethyl carboxamido adenosine; PIA: phenylisopropyl adenosine.

Ap_4A but with lower affinity as observed for the IC_{50} and K_i values. The displacer capacity at the low affinity binding site also presented the range order of P_{2y} receptors. The existence of two different binding sites for Ap_4A is confirmed by these results and the nH value which is close to 0.5 for these compounds.

The agonist ligands for P_1 receptors were **R**-PIA which is more specific for the A_1 receptor and NECA which is more selective for the A_2 receptor. The binding displacement curves for these compounds did not present the two-stepped shape (Figure 5). The displacement values IC_{50} and K_i were six orders and two orders of magnitude lower when compared with the high affinity and low affinity binding displacement for Ap_4A and Ap_5A (Table 1). There were no differences between NECA and **R**-PIA as displacers for Ap_4A. The Hill number obtained for both compounds was 1.

Discussion

The presence of two high affinity binding sites for Ap_4A and related compounds is described in this work, on cultured bovine chromaffin cells. Thus, the presence and release of these compounds from secretory granules reaches a physiological significance; which provides information about their inhibitory control of exocytosis in the same cellular model (Rodriguez del Castillo *et al.*, 1988; Castro *et al.*, 1990; Pintor *et al.*, 1991).

The first binding site for Ap_4A, had a very high affinity K_d value of 0.08 nM (8×10^{-11} M), the highest affinity reported for a nucleotide binding site, although Ap_4A is a naturally occurring compound. The second high affinity binding site had a K_d of 5.6 nM (5×10^{-9} M) which is closer to the values reported in the literature (Williams, 1987; Cooper *et al.*, 1989; Keppens *et al.*, 1989). The coincidence in the same cell of very high and high affinity binding sites has also been shown for peptidic hormones (Lipkin *et al.*, 1986; Shapiro *et al.*, 1986; Betschart *et al.*, 1986; Delicado *et al.*, 1987).

The estimated values of very high and high affinity binding sites for Ap_4A for one chromaffin cell were 5420 ± 450 and 70,000, respectively. Similar estimated values are described for very high and high affinity insulin receptors in mammalian cells. The percentage of the very high affinity binding sites is close to 10% of the total.

The displacement studies indicate that the Ap_4A, Ap_5A and Ap_6A have identical or very similar affinities ($K_i = 1.3 \times 10^{-11}$) for the very high affinity binding sites. However, Ap_3A shows an affinity two orders of magnitude lower ($K_i = 1 \times 10^{-9}$ M). Nevertheless, the second binding site is more specifically displaced by Ap_4A and Ap_5A than by Ap_6A, which presents an effect similar to the Ap_3A (Table 1). In this cellular model, diadenosine polyphosphates have an inhibitory action on nicotine-evoked release (Castro *et al.*, 1990). Their effectiveness corresponds to a potency order of $Ap_4A > Ap_5A \simeq Ap_3A$. Ap_4A is the most active compound, but other factors in addition to binding have to be considered to explain the behaviour of Ap_3A and Ap_5A. There are no data available about Ap_6A effects on catecholamine secretion.

ATP analogues displace the Ap_4A binding with the potency order of the P_{2y} receptors, adenosine 5'-O-(2 thiodiphosphate) (ADP-β-S) > 5'-adenylyl-imidodiphosphate (AMP-PNP) > α,β-Me-ATP, in both binding sites. The K_i values ranged from 7×10^{-11} to 7×10^{-10} M for the high affinity displacement and from 1×10^{-7} to 9×10^{-7} M for the second binding site (Gordon, 1986; Burnstock & Kennedy, 1985; Burnstock, 1989; Cooper *et al.*, 1989; Cusack & Hourani, 1990).

R-PIA and NECA exhibited the same displacement capacity with a very low affinity ($K_i = 1 \times 10^{-5}$ M). Although chromaffin cells and their tumoral homologue, the PC 12, seem to present the A_2 purinoceptor, (Delicado *et al.*, 1990; Noronha-Blob *et al.*, 1986; Williams, 1987; Williams *et al.*, 1987).

The presence of these two high affinity binding sites for Ap_4A deserves special considerations. The first is related to the extracellular levels of Ap_4A and Ap_5A reached after carbachol-induced release. Chromaffin cells can release about 27 pmol/1 $\times 10^6$ cells of both compounds, the volume of 10^6 chromaffin cells being about 1 μl (Pintor *et al.*, 1991). Thus, if the extracellular volume of distribution is similar to (or less than) the intracellular volume, the concentration reached ought to be 27 μM at the surrounding area of stimulated cells under the most unfavourable circumstances. In this situation both sites would be saturated by the ligand. This could also be the case for a synaptic cleft. Additionally the Ap_4A or Ap_5A concentration would maintain their actions through the very high affinity receptor even after 10^5–10^6 times dilution, as long as diadenosine polyphosphates are diffusing. Moreover, target cells endowed with the very high affinity receptor could also respond to these compounds, even if they are located far from the releasing sites. This aspect takes special relevance at the vascular system where the effects of Ap_4A have been found (Busse *et al.*, 1988).

Another important consideration related to the previously noted is whether Ap_4A and Ap_5A are the natural ligands for the P_{2y} receptors or a different species of purinoceptors. Pharmacological studies in the vas deferens and urinary bladder suggest a possible action of diadenosine polyphosphates through P_{2x} receptors, but many of their actions cannot be explained in terms of activation of the already known purinoceptors. Hoyle suggests that classes of receptors for adenine dinucleotides and nucleotides in general, are yet to be discovered (Hoyle, 1990).

In conclusion, our studies demonstrate the presence of very high and high affinity receptors for Ap_4A in a homogeneous neural cell population, the chromaffin cells, with a P_{2y} profile in displacement binding studies and in the absence of ectophosphodiesterase activity. A broad and careful study in other homogeneous and well defined cellular systems is necessary for further understanding of the physiological relevance of these natural compounds.

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The effects of soman on the electrical properties and excitability of bullfrog sympathetic ganglion neurones

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1 The effects of soman (0.1–10 μM), an irreversible inhibitor of acetylcholinesterase (AChE), were examined on the electrical properties of ganglion neurones of the paravertebral sympathetic chain of the bullfrog, *Rana catesbeiana*.

2 Soman (10 μM) depolarized 29 of 35 (83%) ganglion neurones studied by 6.4 $\pm$ 0.65 mV within 10 min of application and reduced the cell input resistance in 9 of 11 neurones examined (82%) to 55 $\pm$ 5.3% of control.

3 Soman (10 μM) significantly reduced the maximum amplitude and the maximum rate of rise of the action potential and the duration, but not the amplitude, of the after-hyperpolarization (AHP) following the action potential elicited by either direct or antidromic stimulation. The maximum rate of fall and the duration of the action potential were not significantly affected by soman. These actions of soman were independent of the agent-induced depolarization. When examined by a single microelectrode voltage clamp, soman reduced the amplitude and the time constant of the current underlying the slow AHP, I_{AHs}.

4 Soman (1–10 μM) produced an increase in neuronal excitability which was evidenced as either an increase in the number of action potentials or a decrease in the interspike interval in response to constant-current depolarizing pulses. The soman-induced increase in excitability occurred independently of both the agent-induced depolarization and the decrease in input resistance, was reversible with washing, was not caused by an inhibition of the M-current and was also recorded in dissociated sympathetic ganglion neurones.

5 The effects of soman on the membrane potential, input resistance and the duration of the AHP but not cell excitability were blocked by pretreatment with atropine (10 μM). Pretreatment with dihydro-β-erythroidine (DHβE) (10 μM) was ineffective in blocking or reversing the effects of soman. These results suggest that the direct actions of soman on the electrical properties of these neurones are mediated by activation of muscarinic receptors.

Keywords: Antiesterases; acetylcholinesterase inhibitors; soman; sympathetic ganglion neurones of bullfrog; afterhyperpolarization; neuronal excitability

Introduction

Most actions of acetylcholinesterase (AChE) inhibitors have traditionally been described as indirect; mediated by the accumulation of acetylcholine at cholinergic synapses (for reviews see, Holmstedt, 1963; Karczmar, 1967; Volle, 1980; Lukomskaya *et al.*, 1980; Dun *et al.*, 1987). However, direct effects of these agents have been observed which were unrelated to their ability to inhibit AChE at nerve-muscle (Feldberg & Vartiainen, 1934; Koppanyi & Karczmar, 1951; Paton & Perry, 1953; Mason, 1962; Riker & Kosay, 1970; McIsaac & Albrecht, 1975; MacDermott *et al.*, 1980; Yarowsky *et al.*, 1984; Mo *et al.*, 1985; Parsons *et al.*, 1987; Sadoshima *et al.*, 1988; Oyama *et al.*, 1989) and ganglion synapses (Eccles, 1944; Riker, 1953; Kuba *et al.*, 1974; Fiekers, 1985a,b; Rao *et al.*, 1987). Previous studies on the actions of irreversible AChE inhibitors on mammalian ganglionic transmission (Yarowsky *et al.*, 1984; Dun *et al.*, 1987) have shown that soman has direct effects unrelated to AChE inhibition depressing ganglionic transmission and altering the electrical properties of mammalian ganglion neurones. Lukomskaya *et al.* (1989) have shown, however, that the actions of irreversible inhibitors of AChE differ in mammalian and amphibian ganglia. The present experiments were undertaken, therefore, to examine the effects of soman on the electrical properties of sympathetic ganglion neurones of the bullfrog. Some of these results have previously been published in abstract form (Heppner & Fiekers, 1988).

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Methods

All experiments were performed *in vitro* on sympathetic ganglion neurones of the bullfrog, *Rana catesbeiana* obtained from Lemberger Co. Inc. (Oshkosh, WI, U.S.A.). The sympathetic chain from the 5th to the 10th ganglion was removed along with spinal nerves 9 and 10 following decapitation. The procedures used for the isolation and preparation of the sympathetic chain have been described previously (Heppner & Fiekers, 1991). Antidromic stimulation (10–20 V; 1–2 ms duration) was accomplished via a suction electrode attached to the 9th and 10th spinal nerves.

Intracellular microelectrode recordings were obtained from large B-type ganglion neurones with 3 M potassium chloride filled microelectrodes (50–100 MΩ). An Axoclamp 2A was used to measure membrane voltage and inject current. The current underlying the slow component of the action potential afterhyperpolarization (I_{AHs}) was recorded with the hybrid clamp which required switching from current-clamp to single electrode voltage clamp following the action potential as previously described (Goh & Pennefather, 1987). Data were stored and analyzed on a Micro PDP/11-23 laboratory computer and an Indec interface and plotted on a Hewlett Packard plotter.

Resting membrane potential was measured as the potential difference between a reference electrode placed in the bath and an intracellular microelectrode. Only neurones with a resting membrane potential of at least −45 mV and an overshooting action potential were used for analysis. Neuronal input resistance was measured by injecting hyperpolarizing constant-current pulses (0.1–0.3 nA; 100–300 ms) before and after the

Table 1 The effect of soman on action potential (AP) configuration in bullfrog sympathetic ganglion neurones

	AP ampl (mV)	*Max. rate of rise* ($V s^{-1}$)	*Max. rate of fall* ($V s^{-1}$)	*AP duration* (ms)
Control	83.3 ± 5.48	140.5 ± 15.4	86.3 ± 14.8	2.0 ± 0.2
Soman	76.8 ± 6.12*	114.4 ± 16.2*	76.6 ± 11.4	2.0 ± 0.2

Values are expressed as means ± s.e.mean. Individual neurones were maintained at the same membrane potential before and after soman addition (10 μM). All values were obtained from 5 neurones maintained between −45 to −55 mV. Action potential duration values were obtained at one-half maximum amplitude.
* Significant at $P \leqslant 0.05$.

addition of soman. Direct action potentials were elicited by either short (2.0 nA; 2 ms) or long (0.1–1.0 nA; 75–300 ms) depolarizing current pulses. Antidromic action potentials were elicited by stimulation of the spinal nerves with brief depolarizing pulses. The preparation was pinned in a special chamber designed for experiments with these agents (Heppner & Fiekers, 1991) and continuously superfused (approximately 1 ml min^{-1}) with a HEPES-buffered control solution of the following composition (mM): NaCl 120, KCl 2.5, $CaCl_2$ 1.8, HEPES 1.0 and adjusted to pH 7.3. Soman (1–10 μM) was applied by continuous superfusion through the bath or by pressure ejection (15–30 s; 3–6 psi) via a 'puffer' pipette with a resistance of 500 kΩ (General Valve Corp., Fairfield, N.J., U.S.A.). The soman concentration in the puffer pipette was 5 μM. Dissociated sympathetic ganglion neurones were prepared by procedures similar to those previously described (Kuffler & Sejnowski, 1983).

Soman was obtained from the U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD., U.S.A. Atropine sulphate was obtained from Sigma Chem. Co. (St. Louis, MO., U.S.A.) and dihydro-β-erythroidine (DHβE) was a gift from Merck Sharp and Dohme Research Laboratories, (Rahway, N.J., U.S.A.). All experiments were performed at room temperature (20–22°C). Average data values are presented as the mean ± standard error of the mean (s.e.mean); n refers to the number of neurones studied. Values were considered statistically significant if $P \leqslant 0.05$ using Student's t test.

Results

Effects of soman on passive membrane properties

Superfusion with concentrations of soman ranging from 0.1–10 μM, produced, within 3 min, a slow and progressive depolarization in 83% (29 of 35 neurones) of the ganglion neurones studied. This depolarization occurred in the absence of either orthodromic or antidromic stimulation. In some neurones the resting membrane potential stabilized within 10 min; others would continue to depolarize 10–15 mV to levels above the threshold for action potential initiation. Once the spontaneous depolarization had begun, the removal of soman from the chamber by washing was ineffective in either reversing or inhibiting the level or rate of depolarization. After a 10 min exposure to 10 μM soman, neurones were significantly depolarized from a mean control value of −55.4 ± 1.41 mV ($n = 29$) to −49.0 ± 1.44 mV ($n = 29$).

In 9 of 11 neurones examined, the input resistance was reduced in soman (10 μM) from a control value of 80.2 ± 11.33 MΩ to 44.4 ± 8.19 MΩ ($n = 9$) (55 ± 5.3% of control; $P \leqslant 0.05$). The soman-induced decrease in the input resistance was accompanied in many neurones by the soman-induced depolarization. Since the membrane leak conductance increases with membrane depolarization, the input resistance was also examined when the membrane potential was maintained at pre-agent levels with current injection. Eight of 13 neurones (62%) demonstrated a decrease in input resistance from a control value of 104.4 ± 15.0 MΩ to 75.1 ± 9.6 MΩ ($P \leqslant 0.05$) when exposed to soman (10 μM). Therefore, the decrease in the input resistance was not entirely dependent on the membrane depolarization. Figure 1a illustrates an experiment in which the input resistance was decreased by 20% in 10 μM soman with the membrane potential maintained at the resting level. The soman-induced change in the input resistance was not reversed with washing.

Effects of soman on action potential configuration

The effects of soman were examined on action potentials elicited by direct and antidromic stimulation. The maximum rate of rise, maximum amplitude, maximum rate of fall, action potential duration and the duration of the after-hyperpolarization (AHP) were examined in neurones with the membrane potential maintained at the resting potential by current injection. Only neurones in which the membrane potential was maintained constant and for which measurements were obtained in both control solution and soman were included in this analysis. Soman significantly decreased the maximum amplitude and the maximum rate of rise without significantly affecting either the maximum rate of fall or the duration of the action potential (Table 1).

Soman increases the excitability of sympathetic neurones

In the majority of ganglion neurones there was little spontaneous synaptic activity and, unless the resting membrane potential was close to threshold, spontaneous action potential generation was infrequent. However, following soman, there was an increase in the number of repetitive action potentials at the anode break of a hyperpolarizing current pulse suggesting that soman increased cell excitability (Heppner & Fiekers, 1988). Figure 1b illustrates the soman-induced increase in neuronal excitability as well as the progressive change in the cell input resistance following soman exposure. The interdependence of these effects is discussed below. Depolarizing step pulses of constant-current amplitude (0.3–1.0 nA; 75 ms) were applied to neurones at several membrane potentials. In control solution these pulses elicited from zero to two single overshooting action potentials followed by an AHP. Exposure to soman (10 μM) produced either an increase in the number of action potentials or a decrease in the time interval between multiple action potentials following these depolarizing step pulses. Of the neurones studied, 50% demonstrated an increase in the number of action potentials and 35% showed a decrease in the interspike interval. The effects of soman on cell excitability but not on the duration of the AHP or membrane potential were reversible with washing for up to 45 min (Figure 1c). To examine the dependence of the soman-induced excitability on membrane potential, we performed similar experiments with the neurones maintained at the resting membrane potential by current-injection before, during and after soman exposure (Figure 1c). Soman (1–10 μM) consistently induced an increase in neuronal excitability in the absence of any change in the resting membrane potential (Figure 1c). In several experiments the membrane potential was restored by current injection following the soman-induced depolarization. There was no difference in the soman-induced excitability whether the decrease in the membrane potential was negated electrotonically either during or following the soman-induced depolarization.

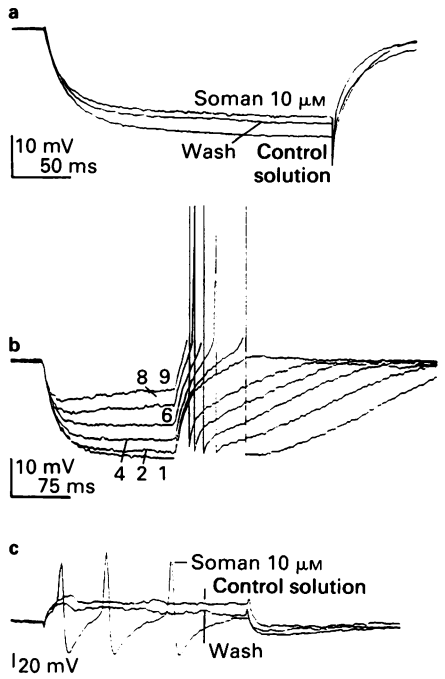

Figure 1 (a) Constant current hyperpolarizing pulses (0.4 nA; 300 ms) were injected into a ganglion cell in control solution, after 10 min in soman (10 μM) and after a 45 min wash. The membrane potential was maintained throughout the experiment at −52 mV with current injection. Soman reduced the input resistance to 80% of control. Each record represents the mean of 10 traces. (b) Constant-current hyperpolarizing pulses (0.4 nA; 225 ms) were injected into another sympathetic neurone in the absence of current injection. Trace 1 was recorded in control solution and subsequent traces were recorded at 2, 4, 6, 8 and 9 min following soman addition to the recording chamber. The membrane potential depolarized in soman from −54 mV in control solution to −48 mV after 9 min in soman (10 μM) but the traces have been superimposed to facilitate comparison. (c) The membrane potential in this neurone was maintained at −40 mV throughout the experiment with current injection. A constant current depolarizing pulse (0.3 nA; 100 ms) was injected into the neurone in control solution, 5 min after the addition of soman (10 μM) and after a 30 min wash. The soman-induced increase in neuronal excitability was reversed with washing. The action potential amplitudes are truncated in (b) and (c).

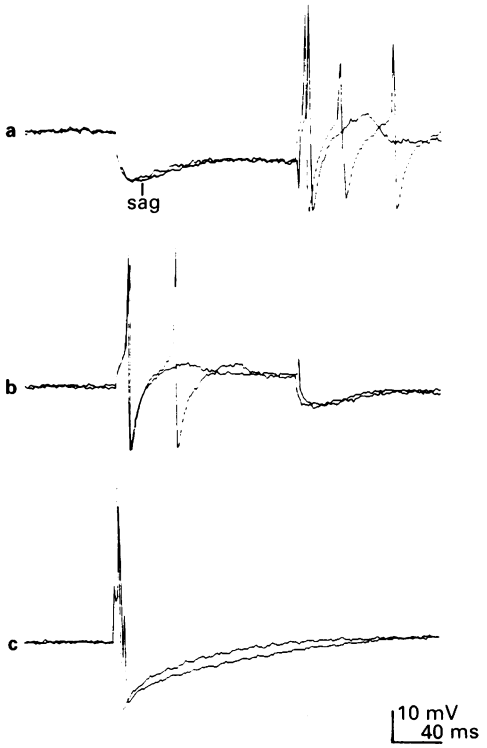

Figure 2 Soman shortens the afterhyperpolarization (AHP) but does not affect the M-current. A series of superimposed recordings from a single neurone before and after exposure to soman (1.0 μM). Constant current hyperpolarizing pulses (0.3 nA; 200 ms) were injected into the neurone. The 'sag' associated with closure of the M-channels was unchanged by soman addition (a); however, soman produced multiple anodal break action potentials at the end of the pulse when compared to the control trace. The membrane potential was maintained by current injection at −40 mV. (b) Constant-current depolarizing pulses (0.2 nA; 200 ms) were injected into the neurone. The control trace shows a single action potential. Following soman several action potentials were recorded. The membrane potential was maintained at −50 mV by current injection. (c) Brief depolarizing current pulses (1.7 nA; 2 ms) evoked an action potential followed by an AHP. Following soman, the AHP was reduced in duration. The membrane potential was maintained at −75 mV. The action potential amplitudes in this figure are truncated.

Effects of soman on the M-current

The M-current is an important regulator of repetitive firing in bullfrog sympathetic ganglion neurones (Adams *et al.*, 1982; Goh & Pennefather, 1987). Several experiments were performed, therefore, to examine the effects of soman on the M-current using both current and voltage clamp recordings. Figure 2 illustrates results obtained in a single neurone in which the membrane potential was maintained with current injection at −40 mV before and after soman addition (1 μM) (superimposed traces). Soman did not alter the 'sag' which represents the closure of the M-channels during constant-current hyperpolarizing pulses (Figure 2a) (Adams *et al.*, 1982). However, neuronal excitability was still induced by soman as evidenced by the increase in the number of anode-break action potentials observed at the end of the pulse (Figure 2a) as well as the increased number of action potentials recorded in response to constant-current depolarizing pulses (Figure 2b). The increase in excitability produced by soman was not the result of a change in the cell input resistance which was unchanged in this neurone. The instantaneous step and the slow relaxation are frequently merged when the membrane potential is maintained with current injection (Adams *et al.*, 1982). Therefore, the effects of soman on the M-current were also determined under voltage clamp (Figure 3). The M-current was evoked by holding the neurone at −35 mV and stepping to −50 mV. This induced an inward relaxation of the current trace which was due to the slow closure of the M-channels. Figure 3 illustrates the amplitude and the kinetics of the M-current before and after soman exposure. Soman in concentrations ranging from 1.0 μM to 10 μM did not significantly alter either the instantaneous or

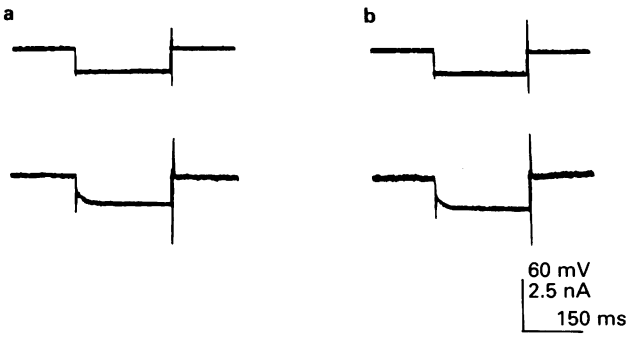

Figure 3 The effects of 10 μM soman on a bullfrog sympathetic neurone recorded under voltage clamp. The neurone was clamped at a holding potential of −35 mV and subjected to 300 ms voltage jumps to −50 mV at 10 s intervals before (a) and during (b) soman superfusion. Voltage records are shown above and current records below in each pair. Soman did not alter either the instantaneous or steady state amplitude of the current. The slow inward current relaxation following the initial ohmic step was not significantly affected by soman.

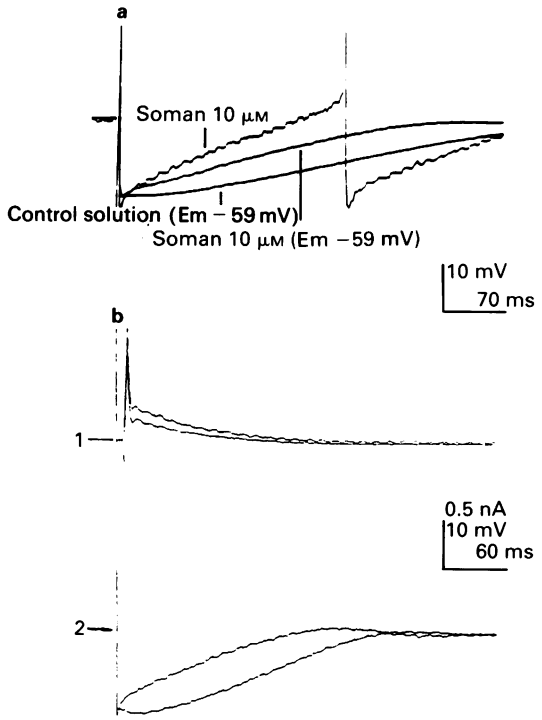

Figure 4 Soman produces an increase in neuronal excitability following antidromic stimulation. (a) Soman produced a decrease in the duration of the afterhyperpolarization (AHP) which was not completely dependent on the resting membrane potential. Soman depolarized this neurone 7 mV from a resting potential of −59 mV to −52 mV and markedly reduced the AHP duration (trace, 10 μM soman). Traces obtained in control solution and soman have been superimposed to facilitate comparison. Returning the neurone to the original resting potential (10 μM soman, Em = −59 mV) by current injection increased the duration of the AHP; however, the duration of the AHP was still reduced in soman. Action potential amplitudes are truncated. The records clamped at −59 mV represent the mean of 10 traces. (b) Soman also decreased both the I_{AHs} recorded with a single electrode voltage clamp (b1) and the duration of the AHP in current-clamp recordings (b2) in a single sympathetic neurone in control solution and following the addition of soman (10 μM). Each record represents the average of 7 individual traces recorded under the same conditions in the same neurone. Current records of the I_{AHs} (b1) were obtained starting at 15 ms after the evoked action potential and were fitted with a single exponential function. Both traces (b1 and b2) were recorded from the same neurone voltage clamped at −45 mV. The action potential amplitudes are truncated. Parameter values for I_{AHs} (b1) were (amplitude and decay time constant): control, 0.5 nA and 94 ms; soman: 0.3 nA and 66 ms. Parameter values for the AHP (b2) were (amplitude and duration): control, 24 mV and 275 ms; soman: 25 mV and 181 ms.

steady state amplitudes during the voltage step. The instantaneous and steady state values, respectively, were: 1.06 ± 0.19 nA and 1.87 ± 0.11 nA in control and 1.09 ± 0.12 nA and 1.78 ± 0.16 nA ($n = 3$) in soman (1.0 μM). The time course of the M-current relaxation examined in several neurones was also not significantly affected by soman. For example, the time constant of the M-current relaxation in one neurone was 81.1 ms in control and 80.4 ms in 10 μM soman.

Soman alters the action potential afterhyperpolarization

Alterations in the amplitude and duration of the AHP following the action potential have previously been shown to influence neuronal excitability (Goh & Pennefather, 1987; Hotson & Prince, 1980; Kawai & Watanabe, 1986). Either antidromic or direct stimulation of neurones elicited an action potential with a relatively long AHP. Soman decreased the duration of the AHP following either direct or antidromic depolarizing pulses (Figures 1b, 2c, 4). Figure 4a demonstrates the effects of 10 μM soman in response to single antidromic stimuli when the membrane potential either was not or was maintained at the resting level by current injection. Although the excitability of the neurone was decreased by the restoration of the membrane potential with current injection, the duration of the AHP was still considerably shorter than control. The peak amplitude of the AHP was not significantly different from the control value (Figure 4a). The mean duration of the AHP in control solution was 430.2 ± 48.7 ms ($n = 10$). Following soman (10 μM) the AHP duration was reduced to 274.3 ± 52.9 ms ($n = 7$) ($P \leqslant 0.001$).

Soman decreases I_{AHP}

The shortening of the AHP in the presence of soman may be a consequence of a reduction in the membrane time constant. Recording the underlying current of the AHP eliminates distortions introduced by changes in capacity and leak currents. We recorded the slowly decaying, calcium dependent, I_{AHs}, with a switching single microelectrode hybrid clamp (Goh & Pennefather, 1987; Tanaka *et al.*, 1986). The currents underlying the action potential are too large and fast to be effectively clamped with a single microelectrode; however, by activating the clamp after the initiation of the repolarizing phase, it is possible to record the majority of the current underlying the slow AHP (Goh & Pennefather, 1987). Recordings were obtained before and after soman exposure from neurones clamped at the resting membrane potential. The peak amplitude of the outward current underlying the I_{AHs} as well as the rate of decay of the I_{AHs} were both significantly reduced by soman (Figure 4b). Both in control and following soman, the decay rates of the I_{AHs} were well described by a single exponential. Soman (10 μM) significantly decreased the amplitude of the I_{AHs} from a control value of 0.69 ± 0.09 nA to 0.45 ± 0.09 nA ($n = 14$; $P \leqslant 0.05$). The decay time constant of the I_{AHs} was significantly reduced from 145.3 ± 15.2 ms to 104.2 ± 14.2 ms in soman ($n = 6$) ($P \leqslant 0.05$).

The effects of soman in the presence of receptor antagonists

The actions of soman on the membrane potential, membrane resistance and the duration of the AHP were examined in the presence of concentrations of specific receptor antagonists sufficient to block completely nicotinic (DHβE, 10 μM) and muscarinic (atropine, 10 μM) cholinoceptors (Kobayashi & Libet, 1970; Jan & Jan, 1982). Isolated ganglion preparations were incubated for 30–60 min with each agent alone or with both agents simultaneously before addition of soman (10 μM). Pretreatment with a combination of atropine (5–10 μM) and DHβE (10 μM) or with atropine (10 μM) alone blocked (1) the soman-induced depolarization, (2) the decrease in membrane resistance and (3) the duration of the AHP. Pretreatment with DHβE alone was ineffective in blocking these actions of soman. These results are summarized in Table 2.

Actions of soman on dissociated neurones

The effects of soman were also examined on dissociated sympathetic ganglion neurones. Depolarizing and hyperpolarizing constant current pulses were injected into neurones to determine cell excitability and input resistance, respectively. In contrast to the results obtained in the intact ganglia, there was no significant change in either the input resistance of the membrane potential of the dissociated neurones when exposed to soman. However, there was a consistent, reversible and reproducible increase in neuronal excitability in the presence of soman (Figure 5). In every neurone, ($n = 10$), soman (5 μM) significantly increased the number of action potentials with constant-current depolarizing pulses. In addition, similar increases in excitability were obtained in these neurones in the presence of DHβE and atropine.

Table 2 The effect of soman on the passive membrane properties in the presence of nicotinic and muscarinic receptor antagonists

	Membrane control (mV)		*AHP duration* (ms)		*Resistance*
	Control	*Soman*	*Control*	*Soman*	(% of control)
Atropine + DHβE	51.8 ± 2.4 (8)	51.9 ± 2.9 (8)	325 ± 66.1 (6)	296 ± 49.9 (6)	103 ± 7.5 (8)
Atropine	56.7 ± 3.5 (4)	55.8 ± 2.8 (4)	436 ± 58.6 (4)	404 ± 52.6 (4)	97 ± 5.5 (4)
DHβE	50.2 ± 3.2 (6)	46.3 ± 2.5 (6)*	434 ± 46.4 (6)	368 ± 53.5 (6)*	69 ± 8.0 (6)*

Values are expressed as mean ± s.e.mean. Individual neurones were maintained at the same membrane potential before and after soman addition (10 μM) to measure AHP duration and input resistance. Number of experiments is shown in parentheses. DHβE = dihydro-β-erythroidine.

* Significantly different from control at $P \leqslant 0.05$.

Discussion

Actions of anticholinesterase agents on membrane potential have been well documented (Mason, 1962; Volle, 1980). A soman-induced depolarization in the rat superior cervical ganglion (SCG) (Yarowsky *et al.*, 1984) and a hyperpolarization in cat parasympathetic ganglion neurones (Kumamoto & Shinnick-Gallagher, 1990) were accompanied by a decrease in neuronal input resistance. In the rabbit, the soman-induced depolarization was present in about half of the neurones studied but was not associated with any change in input resistance (Dun *et al.*, 1987). In the present experiments, the depolarization produced by soman was accompanied by a decrease in input resistance. Previous studies have shown that preganglionic stimulation is required for armin to depolarize ganglion neurones in the frog but not the rabbit (Lukomskaya *et al.*, 1989). However, in our experiments we observed a depolarization in the absence of orthodromic stimulation in the intact ganglia, but not in dissociated neurones. This suggests either that the soman-induced depolarization in the bullfrog is mediated by a presynaptic action as suggested in the rabbit (Dun *et al.*, 1987) or that the postsynaptic site with which soman interacts is no longer present following enzymatic dissociation.

In addition to an effect on the membrane potential, Yarowsky *et al.* (1984) demonstrated direct effects of soman on the input resistance and concluded that these postjunctional effects were responsible for changes in the parameters of the action potential in rat SCG. Although these experiments were not performed with the membrane potential maintained by current injection, Yarowsky *et al.* (1984) indicated that the change in AHP duration could not be fully explained by the decrease in input resistance. Our results demonstrate that soman has similar actions on the electrical properties of bullfrog sympathetic neurones and further indicate that the decrease in the rate of rise, maximum amplitude and AHP duration can occur independently of changes in the resting membrane potential. These results also suggest that soman has actions on the voltage-gated conductances activated during the action potential as well as conductances mediating the AHP. This is not a general property of antiesterase agents, however, since physostigmine did not affect the electrical properties of rabbit SCG ganglia in concentrations sufficient to block receptor-mediated responses (Mo *et al.*, 1985) and diisopropylfluorophosphate produces a depolarization without alterations in the electrical properties of rat SCG neurones (Yarowsky *et al.*, 1984).

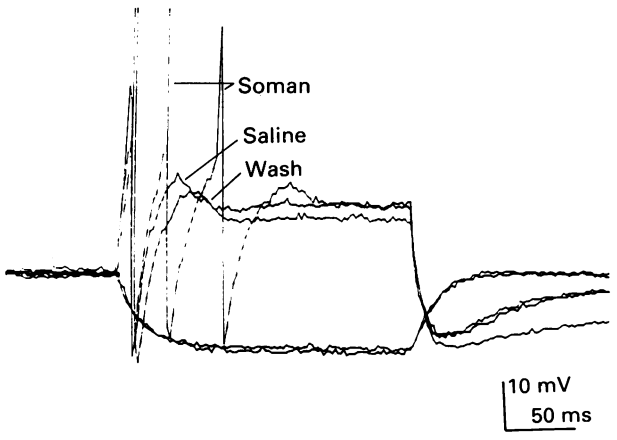

Figure 5 Soman increases neuronal excitability in dissociated ganglion neurones. Puffer applications of soman to dissociated neurones increased cell excitability with constant current depolarizing pulses (0.45 mV; 200 ms). A single action potential was evoked in control solution and during a wash; three action potentials were evoked in soman (5 μM). Constant-current hyperpolarizing pulses (0.1 mV; 200 ms) were also injected in control solution and in soman to monitor changes in input resistance. The increase in excitability produced by soman occurred in the absence of a significant change in the input resistance. There was no change in the resting membrane potential of this neurone (−51 mV) before or after soman application. The action potential amplitudes are truncated in this figure.

An increase in neuronal excitability produced by antiesterase agents has been attributed to an increase in the amplitude and duration of the excitatory postsynaptic potential (e.p.s.p.), due to AChE inhibition, so that repetitive action potentials are elicited with a single stimulus (Riker & Guerrero, 1968; Dun *et al.*, 1987; Lukomskaya *et al.*, 1988; Fiekers & Heppner, 1988). Alternatively, a presynaptic effect of the antiesterases has been proposed to account for stimulus-bound repetitive activity in bullfrog ganglia produced by neostigmine (Riker & Guerrero, 1968; 1970). Our results differ from these previous results because (1) in Riker & Guerrero's studies there was no increase in neuronal excitability with either single, direct or antidromic stimulation, (2) we did not use orthodromic stimulation in the present study so there was no increased residual e.p.s.p. and (3) a reproducible increase in neuronal excitability could be obtained in the presence of soman in dissociated sympathetic neurones.

Several lines of evidence suggest that the increase in neuronal excitability produced by soman can occur independently of changes in the membrane potential and input resistance. The soman-induced increase in neuronal excitability and membrane depolarization were observed in a similar proportion of neurones studied (~80%); however, these effects were not causally related since the excitability induced by soman was present in neurones in which the membrane potential was maintained by current injection and the neuronal excitability remained following restoration of the membrane potential after the soman-induced depolarization. In addition, soman increased the excitability in dissociated ganglion neurones in which no depolarization or decrease in input resistance was recorded. Furthermore, the soman-induced changes in membrane potential, input resistance and AHP duration but not excitability were blocked by atropine pretreatment. These results suggest that the mechanisms underlying these effects of soman are different and that the changes in the electrical properties produced by soman are primarily mediated via muscarinic receptor activation.

In bullfrog sympathetic neurones, the AHP and the M-current are two mechanisms known to regulate excitability (Busis & Weight, 1976; Kuba & Koketsu, 1978; Adams *et al.*, 1982; Goh & Pennefather, 1987). The increase in excitability

produced by soman was still recorded in the absence of an effect of soman on the M-current suggesting that M-current inhibition was not required for the increased excitability. The amplitude of the AHP also depends on currents repolarizing the action potential (Akasu *et al.*, 1983; Lancaster & Pennefather, 1987), and soman did not significantly decrease the initial amplitude of the AHP. In addition, soman does not alter either the rate of decay or the duration of the action potential. These results suggest that soman does not significantly affect potassium conductances activated during the action potential.

The AHP regulates the excitability in many peripheral and central neurones (Krnjevic & Lisiewicz, 1972; Barratt & Barratt, 1976; McAfee & Yarowsky, 1979; Morita *et al.*, 1982; North & Tokimasa, 1983; Madison & Nicoll, 1986). In the bullfrog the AHP is generated by the activation of a calcium-dependent potassium conductance (Kuba & Koketsu, 1978; Kuba *et al.*, 1983). The duration of the AHP, therefore, depends on factors which regulate cytosolic calcium concentration. The intracellular calcium required for the AHP in bullfrog sympathetic neurones is believed to originate from calcium influx during the action potential (Tanaka & Kuba, 1987). Measurements of the amplitude and decay of the I_{AHs} can indicate sites for the actions of agents affecting the AHP. For example, charybdotoxin, apamin and (+)-tubocurarine block the I_{AHs} without altering the decay of the current or action potential parameters (Tanaka *et al.*, 1986; Kawai & Watanabe, 1986; Nohmi & Kuba, 1984; Goh & Pennefather, 1987). Soman, however, decreased both the amplitude and decay time constant of the I_{AHs} suggesting that soman not only decreases calcium entry during the action potential but also modifies calcium removal within the cytoplasm of these neurones. Soman decreased the rate of rise and maximum amplitude of the action potential which may produce a decrease in calcium entry during the action potential which could contribute to a decrease in AHP duration. Preliminary experiments support the hypothesis that the decrease in the amplitude and duration of the I_{AHs} by soman may be a consequence of a reduction in calcium influx during the action potential (Heppner & Fiekers, 1989). Soman decreases the duration of barium-dependent action potentials approximately 50% at concentrations of soman ranging from 0.1 μM–1.0 μM (Heppner & Fiekers, 1989).

Muscarinic activation alters voltage-gated channels (Kuba & Koketsu, 1975; Kuba & Koketsu, 1978; Akasu & Koketsu, 1982; Tokimasa, 1985) and depresses calcium-dependent action potentials and the AHP in bullfrog sympathetic ganglion neurones (Akasu & Koketsu, 1982; Tokimasa, 1985). The increase in excitability observed in the present experiments is, however, unrelated to either (1) a prejunctional action, (2) a consequence of ACh accumulation or (3) muscarinic activation since it is not blocked by atropine or DHβE pretreatment and occurs independently of changes in the electrical properties of the neurone. These actions of soman in increasing neuronal excitability and decreasing the AHP may contribute to the central toxicity of soman (Baldisseria & Gustaffson, 1974; Van Meter *et al.*, 1978; Karczmar, 1985).

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In vitro vascular effects of cicletanine in pregnancy-induced hypertension

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1 The vascular effects of cicletanine have been studied *in vitro* on ring preparations of inferior epigastric arteries from normotensive human females and human females with pregnancy-induced hypertension (preeclampsia).

2 Cicletanine (10^{-7}–10^{-3} M) elicited concentration-dependent relaxation of vessels precontracted with 10^{-7} M noradrenaline (NA) or 60 mM K^+ but was more potent in the former. Relaxation was significantly greater in rings from preeclamptic patients and was uninfluenced by endothelium removal.

3 The intracellular Ca-dependent contractile responses to 10^{-5} M NA in Ca-free medium as well as the subsequent extracellular Ca-dependent contractions (on restoration of external Ca) were significantly attenuated dose-dependently by cicletanine (10^{-5} M, 3×10^{-4} M) in arterial rings from both normotensive and preeclamptic patients. Cicletanine also relaxed rings precontracted by 25 mM K^+ but was ineffective against 80 mM K^+-induced contractions.

4 The inhibition of intracellular Ca-dependent contractions was significantly greater in rings from preeclamptic than from normotensive patients whereas extracellular Ca-dependent contractions were comparably inhibited in both groups. Nifedipine, on the other hand, had little effect on the intracellular Ca-dependent contractions but significantly depressed extracellular Ca-dependent contractions.

5 Cicletanine-induced relaxation was uninfluenced by pretreatment with propranolol, ouabain, tetraethylammonium, procaine, indomethacin, cimetidine or tetrodotoxin but was antagonized by glibenclamide.

6 The results show that cicletanine inhibits contractile responses of human isolated inferior epigastric arteries by a mechanism unrelated to endothelial factors but associated with inhibition of calcium metabolism. An action of cicletanine on glibenclamide-sensitive K^+ channels is also suggested. Cicletanine-induced inhibition was significantly greater in arteries from preclamptic patients.

Keywords: Cicletanine; pregnancy-induced hypertension; calcium; human epigastric arteries; vascular smooth muscle; endothelium

Introduction

The vascular effects of cicletanine, an antihypertensive fuorpyridine compound, have been described in a variety of animal (Malherbe *et al.*, 1988; Auguet *et al.*, 1988; Bukoski *et al.*, 1989; Ebeigbe *et al.*, 1989) and human (Schoeffter & Godfraind, 1988; 1989) smooth muscles. Cicletanine competitively antagonizes hisamine H_1-receptor-mediated contractions in the guinea-pig isolated ileum (Schoeffter *et al.*, 1987), rabbit mesenteric artery (Ebeigbe *et al.*, 1989), and human coronary, internal mammary artery and saphenous vein (Schoeffter & Godfraind, 1988; 1989). Also, cicletanine-induced inhibition of contractile responses in rabbit mesenteric arterial and aortic smooth muscles (Ebeigbe *et al.*, 1989) have been associated with interference with calcium metabolism within the vascular smooth muscle cells.

In vitro studies on the effects of cicletanine on arterial smooth muscle contractions in the hypertensive state have concentrated on animal models (Malherbe *et al.*, 1988; Auguet *et al.*, 1988; Bukoski *et al.*, 1989). The present paper describes our observations on the vascular effects of cicletanine on isolated epigastric arteries from patients suffering from pregnancy-induced hypertension (preeclampsia). Recent reports from our laboratory have shown that the responsiveness of arterial smooth muscles from humans with pregnancy-induced hypertension bears a striking resemblance to observations in vascular smooth muscle from animals with various forms of experimental hypertension: blood vessels from preeclamptic patients appear to possess vascular membrane defects e.g. enhanced activity of the potential-sensitive Ca^{2+} channels (Ebeigbe *et al.*, 1987), attenuated Na-K ATPase enzyme activity (Ebeigbe *et al.*, 1985; Ezimokhai & Ebeigbe, 1985; Ebeigbe & Ezimokhai, 1988) as well as other functional and morphological abnormalities (Aalkjaer *et al.*, 1985). These render the smooth muscle cells hypersensitive to pressor agents as reported for various forms of experimental hypertension (Webb & Bohr, 1981; Aoki *et al.*, 1982).

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Methods

Subjects

Two groups of subjects (patients attending the University of Benin Teaching Hospital, Benin City, Nigeria) were studied: (a) Control normotensive women, who in the third trimester of normal pregnancy, delivered following elective or emergency caesarean section as part of their obstetric management; (b) Preeclamptic mothers who were proteinuric and demonstrated persistent elevation of blood pressure to at least 140/90 mmHg in the third trimester of pregnancy, having been normotensive in the first and second trimesters; delivery was also either by elective or emergency caesarean section.

All patients were given general anaesthesia induced by thiopentone with suxamethonium and maintained by a mixture of N_2O and O_2. Transverse suprapubic incision was employed in all patients. Informed consent from the patient as well as approval of the ethical committee of the University of Benin Medical School was obtained to excise a segment of the inferior epigastric artery for the study. On excision, the arteries

were immediately placed in physiological salt solution (PSS) and transported to the laboratory.

Preparation of tissue

With the aid of dissecting microscope, the arteries were carefully cleaned of adhering connective tissues and cut into rings (internal diameter, 1.5–2.5 mm; 2–3 mm long). The rings were carefully mounted between two L-shaped stainless steel holders in 20 ml organ baths containing PSS. The composition of the PSS was (mM): NaCl 119.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 1.6, $CaNa_2$ EDTA 0.03 and glucose 11.5. The medium was bubbled continously with a 95% O_2, 5% CO_2 gas mixture. pH of the solution was 7.4 and temperature was maintained thermostatically at 37°C. Isometric contractions were recorded with Grass FT-03 transducers and the responses were displayed on a Grass model 7 10-channel polygraph. An equilibration period of 2 h was allowed before the start of experiments. Further details of the experimental set-up have been described previously (Ezimokhai & Ebeigbe, 1985; Ebeigbe *et al.*, 1987).

Procedure

Relaxant effect of cicletanine Arterial rings were contracted with 10^{-7} M noradrenaline (NA) or 60 mM K^+ PSS. When the contractile responses were stable, cicletanine was added to the bath cumulatively; the resultant relaxation responses were expressed as a percentage of the initial tension. In other experiments, the relaxant effect of cicletanine was also studied following precontraction induced by 25 or 80 mM K^+ PSS.

Influence of endothelium The relaxant effect of cicletanine following pre-contraction induced by 10^{-7} M NA was examined on intact as well as arterial rings without endothelium. Endothelium removal was effected mechanically (Furchgott & Zawadzki, 1980) by gently rubbing the intimal surface of the vessels. The effectiveness of the procedure for removal of the endothelium was confirmed by the failure of acetylcholine to relax NA (10^{-7} M)-precontracted rubbed arteries.

Intra- and extracellular calcium-dependent contractions Maximal contractile responses to NA (10^{-5} M) were assessed in control PSS and following 15 min exposure to a nominally Ca-free medium. The phasic NA contraction in Ca-free medium was presumed to be due to mobilization of calcium from intracellular stores (Bohr, 1973; Dube *et al.*, 1987; Auguet *et al.*, 1988); whereas restoration of Ca following the intracellular Ca-dependent contraction caused a sustained extracellular Ca-dependent (tonic) component. In Ca-restoration experiments, increases in Ca concentration caused graded contractions, with maximum response at 3.6 mM (not shown); in the results presented a submaximal level (3.0 mM) of Ca was used. The influence of varying concentrations of cicletanine and nifedipine on both components of NA-induced contractions was assessed following application of the antagonists simultaneously with the change to Ca-free PSS (the antagonists were maintained throughout the duration of both components of the contractions).

Influence of drugs on cicletanine-induced relaxation In control experiments, arterial rings from normotensive patients were exposed to 5×10^{-6} M (EC_{50}) cicletanine following NA (10^{-7} M)-induced precontraction. The magnitude of the cicletanine relaxation was estimated as a percentage of the initial NA contraction. The influence of various drugs on the cicletanine relaxation was assessed by applying the agents 15 min before NA stimulation.

The drugs used were: cicletanine hydrochloride (racemate, IHB Research Laboratories, Le Plessis Robinson, France); histamine dihydrochloride (Merck AG, Germany); noradrenaline bitartrate (Fluka AG, Bucks, Switzerland); propranolol hydrochloride, ouabain, tetraethylammonium chloride, procaine hydrochloride, indomethacin, cimetidine, tetrodotoxin (Sigma); nifedipine (Bayer Leverkusen, FRG). Glibenclamide was a gift from Professor A.H. Weston (Manchester, UK).

Cicletanine was prepared as a stock solution (10^{-1} M) in absolute ethanol and kept at 4°C. Stock solutions of nifedipine (10^{-2} M) were prepared in absolute ethanol and further dilutions made with distilled water. Experiments with nifedipine were carried out in the dark to avoid photoinactivation of the drug. Indomethacin was dissolved in 50 mM Na_2CO_3. High-K^+ PSS was prepared by equimolar substitution of NaCl with KCl. Ca-free PSS contained no added calcium. All chemicals were of 'analytical reagent' grade.

Statistics

Values are expressed as means ± s.e.mean, IC_{50} (concentration producing 50% inhibition) values were computed by use of a programme for logit transformation of dose-response curves. Statistical comparison of means was made by Student's t test; a P value less than 0.05 was considered significant. n denotes the number of patients studied; 5 rings were prepared from one arterial segment obtained from each patient.

Results

Relaxant effects of cicletanine

The contractile responses to 10^{-7} M NA and 60 mM K^+ in control (normotensive) arterial rings were 2.8–0.3 g, $n = 12$ and 2.8 ± 0.1 g, $n = 10$, respectively. In rings from preeclamptic patients, contractile responses to both NA ($n = 12$) and 60 mM K^+ ($n = 9$) were enhanced (Table 1). Cicletanine (10^{-7}–10^{-3} M) relaxed both NA- and 60 mM K^+ precontracted rings concentration-dependently (Figure 1). Arterial rings from preeclamptic patients were significantly more sensitive to cicletanine than those from normotensive (Table 1). In other experiments on arterial rings from normotensive patients ($n = 3$), cicletanine caused concentration-dependent relaxation of the 25 mM K^+-induced contraction but was ineffective against the 80 mM K^+-induced contraction (Figure 2).

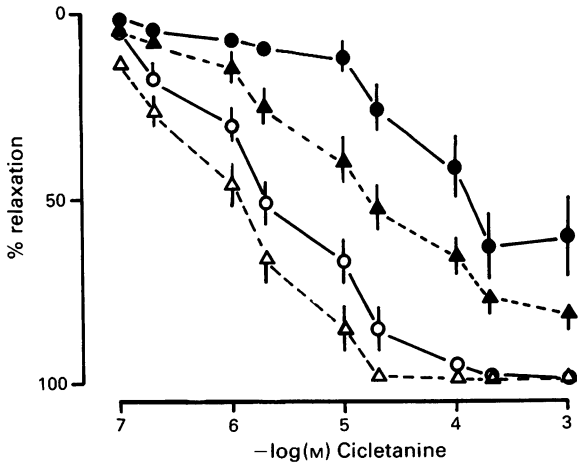

Figure 1 Concentration-dependent cicletanine-induced relaxation of 10^{-7} M noradrenaline- (open symbols) and 60 mM K^+-(closed symbols) induced contractions in rings of inferior epigastric arteries from normotensive (circles, $n = 12$) and pregnancy-induced hypertensive (triangles, $n = 12$) patients. Points are means with s.e.mean shown by vertical lines. Asterisks denote significant difference from controls.

Table 1 Magnitudes of contraction (g) in response to 10^{-7} M noradrenaline (NA) and 60 mM K^+ and IC_{50} (M) values for cicletanine-induced relaxation in inferior epigastric arterial rings from normotensive and preeclamptic patients

	10^{-7} M NA		*60 mM K^+*	
	Normotensive	*Preeclamptic*	*Normotensive*	*Preeclamptic*
Tension	2.8 ± 0.3	3.2 ± 0.1*	2.8 ± 0.6	3.2 ± 0.1*
IC_{50}	3.2(±0.5) × 10^{-6}	1.9(±0.6) × 10^{-6}**	1.8(±1.4) × 10^{-4}	4.1(±0.2) × 10^{-5}**
n	12	12	10	9

Means ± s.e.mean. * $P < 0.001$ and ** $P < 0.05$.

Influence of endothelium

In intact preparations from normotensive patients, 10^{-6} M ACh caused relaxation of 10^{-7} M NA-induced precontraction ($n = 12$) by 78.6 ± 3.2%. ACh-induced relaxation was significantly lower in rings from preclamptic patients (46.3 ± 5.2%, $n = 10$).

Contractile responses to 10^{-7} M NA were enhanced following endothelium removal: in rings, from normotensive patients, tension in the presence and absence of endothelium was 2.8 ± 0.2 g ($n = 12$) and 3.3 ± 0.0 g ($n = 9$), respectively; in rings from preeclamptic patients, the respective tension values in the presence and absence of endothelium were 3.0 ± 0.0 g ($n = 10$) and 3.5 ± 0.0 g ($n = 10$).

Relaxation induced by cicletanine (10^{-7}–10^{-3} M) was uninfluenced by endothelium-removal (data not shown).

Intra- and extracellular Ca^{2+}-dependent contractions

The maximal (10^{-5} M) NA-induced contraction in normal PSS for rings from normotensive ($n = 14$) and preeclamptic ($n = 16$) patients were 3.6 ± 0.1 and 3.8 ± 0.0 g respectively. The magnitude of the phasic, intracellular Ca^{2+}-dependent contraction in rings from normotensive ($n = 14$) and preeclamptic ($n = 16$) patients were 1.0 ± 0.4 and 1.5 ± 0.9 g respectively. The respective extracellular Ca-dependent contraction values for rings from normotensive and preeclamptic patients were 3.9 ± 0.5 and 3.9 ± 0.3 g.

Figure 3 illustrates typical observations on the effect of cicletanine and nifedipine on intra- and extracellular Ca-dependent contractions. Cicletanine (10^{-5} and 3×10^{-5} M) inhibited both intra- and extracellular Ca-dependent contractions in rings from both patient groups. The inhibition of intracellular Ca-dependent contractions was significantly greater in rings from preclamptic patients (Figure 4) whereas

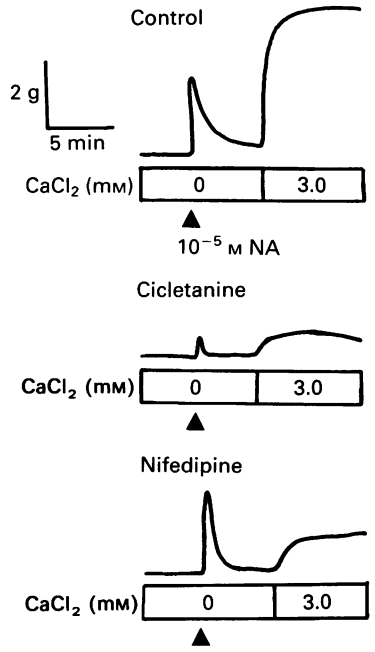

Figure 3 Representative tracings showing contractile responses of rings of epigastric arteries from normotensive patients to 10^{-5} M noradrenaline (NA) following 15 min exposure to Ca-free PSS in the absence or presence of cicletanine or nifedipine. The phasic contractile response to NA in Ca-free solution and the subsequent tonic contraction on restoration of external calcium represent (respectively), intracellular Ca-dependent and extracellular Ca-dependent contractions.

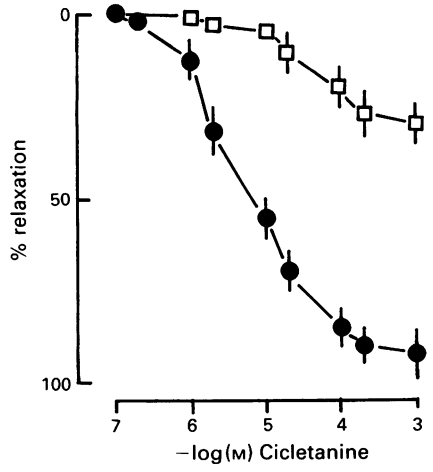

Figure 2 Cicletanine-induced relaxation in ring preparations of epigastric artery from normotensive patients following precontraction induced by 25 mM (●) or 80 mM (□) K^+. Means with s.e.mean; $n = 3$.

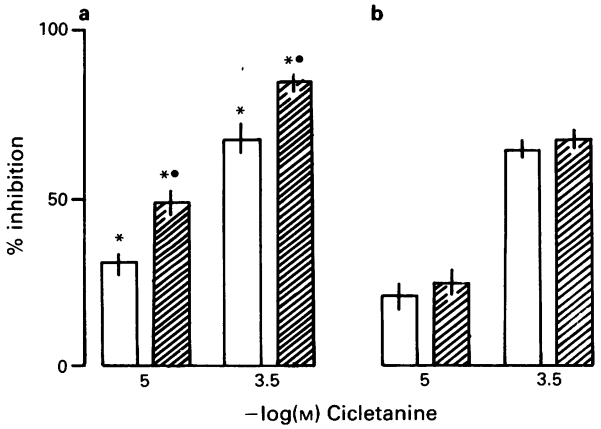

Figure 4 Summary of experiments described in Figure 3. Cicletanine (10^{-5}, 3×10^{-4} M) attenuated both intracellular (a) and extracellular (b) Ca-dependent contractions in arteries from normotensive (open columns, $n = 14$) and preeclamptic (hatched columns, $n = 16$) patients. Cicletanine-induced inhibition was significantly greater on intracellular Ca-dependent contractions (* $P < 0.001$) and was greater in rings from preeclamptic than from normotensive patients (●, $P < 0.05$).

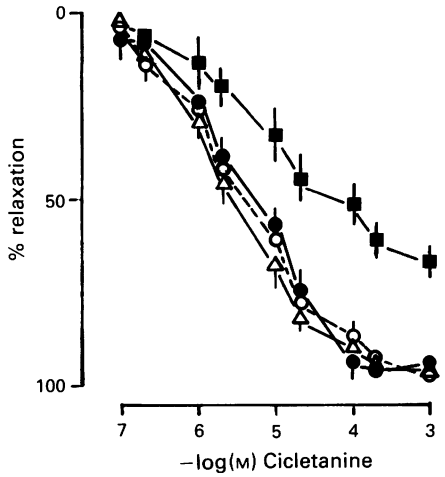

Figure 5 Attenuation by 10^{-6} M glibenclamide (■, $n = 5$) of cicletanine-induced relaxation following 10^{-7} M noradrenaline precontraction and the lack of effect of 10^{-2} M tetraethylammonium (○, $n = 3$) and 10^{-3} M procaine (△, $n = 3$) in rings from normotensive patients. (●) denotes control ($n = 12$). Means with s.e.mean shown by vertical lines.

the magnitudes of inhibition of extracellular Ca-dependent contractions were comparable in rings from normotensive and preeclamptic patients. Contrary to the observation with cicletanine, in rings from normotensive patients, nifedipine (10^{-6} M, $n = 5$) was relatively ineffective against the intracellular Ca-dependent contractions whereas the extracellular Ca-dependent contractions were inhibited by 79.4 ± 5.2%. The concentration of nifedipine used completely antagonized 60 mM K^+-induced contractions (data not shown).

Action of drugs on cicletanine-induced relaxation

The effect of drugs on cicletanine-induced relaxation was studied on arterial rings from normotensive patients. In control experiments ($n = 6$), 5×10^{-6} M cicletanine elicited relaxation of 10^{-7} M NA-induced contractions by 50.4 ± 3.2%.

Exposure of the rings to 10^{-6} M propranolol ($n = 4$), 10^{-5} M ouabain ($n = 3$), 10^{-2} M tetraethylammonium chloride (TEA, $n = 3$), 10^{-3} M procaine ($n = 2$), 10^{-6} M indomethacin ($n = 4$) 10^{-6} M cimetidine ($n = 3$) or 10^{-6} M tetrodotoxin ($n = 3$) did not signifiicantly alter the magnitude of cicletanine-induced relaxation whereas 10^{-6} M glibeclamide ($n = 5$) significantly inhibited the relaxation response; magnitude of relaxation in presence of glibenclamide was 22.3 ± 2.9%. Figure 5 illustrates observations made with K^+ channel blockers, showing the lack of effect of TEA and procaine and the inhibitory effect of glibenclamide on the concentration-dependent relaxation to cicletanine.

Discussion

The goal of the present study was to characterize the mechanism of cicletanine-induced relaxation of arterial smooth muscle in pregnancy-induced hypertension (preeclampsia). Previous *in vitro* vascular studies on cicletanine in the hypertensive state have employed animal (mainly rat) models (Malherbe *et al.*, 1988; Auguet *et al.*, 1988; Bukoski *et al.*, 1989). Recent work from our laboratory (Ebeigbe *et al.*, 1985; 1986; Ezimokhai & Ebeigbe, 1985; Ebeigbe *et al.*, 1987; Ebeigbe & Ezimokhai, 1988), has shown that studies on vascular smooth muscle in preeclampsia afford an alternative method for assessing vascular disorders in hypertension.

In the present study, we observed that cicletanine-induced relaxation was significantly greater in arteries from preeclamptic patients than from normotensive patients (Figure 1). The effect of cicletanine was also observed to be greater on NA-precontracted than on K^+-precontracted rings. These results suggest a greater susceptibility of arteries from preeclamptic patients to cicletanine action via mechanisms related (perhaps) to receptor activation.

The finding of unchanged cicletanine response following endothelium-removal, clearly shows that the mechanism of action of cicletanine is unrelated to the release of endothelium-derived relaxant factor(s). Comparable lack of endothelial factors in cicletanine-induced relaxation has been reported for rabbit mesenteric arteries (Ebeigbe *et al.*, 1989).

Agonist-induced contractions of vascular smooth muscles can be clearly separable into intracellular- and extracellular Ca^{2+}-dependent components (Bohr, 1973; Bolton, 1979; Ebeigbe, 1982; Dube *et al.*, 1987). The protocol illustrated in Figure 3 permits an assessment of intracellular Ca-dependent and extracellular Ca-dependent contractions (Dube *et al.*, 1987; Auguet *et al.*, 1988). Whereas the Ca^{2+} entry blocker, nifedipine, had little effect on intracellular Ca-dependent and significantly attenuated extracellular Ca-dependent contractions (Figure 3), cicletanine attenuated both components of the contractions, concentration-dependently (Figures 3 and 4). The greater inhibitory effect of cicletanine on intracellular Ca-dependent contractions in rings from preeclamptic patients and the comparable magnitudes of inhibition of extracellular Ca-dependent contractions in rings from both normotensive and preeclamptic patients suggest that the cicletanine action is preferably intracellular. Studies on spontaneously hypertensive (SHR) and Wistar Kyoto (WKY) rats, in which Fura-2 was used to assess intracellular Ca^{2+} mobilization (Bukoski *et al.*, 1989) have also shown a greater inhibition by cicletanine of intracellular Ca^{2+} release in the SHR.

The differential effect of cicletanine on NA and high-K^+ contractions is perhaps explicable in terms of their requirements of intra- or extracellular Ca pools for tension development: NA mobilizes both Ca pools, whereas high-K^+ contractions are dependent on the extracellular pool (Bolton, 1979; Ebeigbe, 1982). Thus the greater sensitivity of NA-induced contractions to cicletanine inhibition (Figure 1) is probably due to the intracellular action of the drug.

The observations that propranolol, cimetidine, ouabain, indomethacin and tetrodotoxin had no significant effect on cicletanine-individual relaxation suggest that the mode of action of cicletanine is unrelated (respectively) to stimulation of β-adrenoceptors, histamine H_2-receptors, Na^+-K^+-ATPase activity, prostaglandin synthesis or stimulation of inhibitory nerves.

Two observations point to a possible involvement of K^+ channel activation in cicletanine-induced relaxation: the relative ineffectiveness of cicletanine in relaxing 80 mM K^+-induced contractions (Figure 2), a criterion for characterizing K^+ channel activation (Weston, 1990), as well as antagonism by glibenclamide of cicletanine-induced relaxation (Figure 5). However, the two other K^+ channel blockers tested (TEA and procaine) did not modify cicletanine-induced relaxation, suggesting that a distinct type of glibenclamide-sensitive K^+ channel mediates the relaxant effect of cicletanine in human epigastric arteries.

In conclusion, the present study shows that cicletanine elicits relaxation of human inferior epigastric arteries. The mechanism of action of cicletanine is unrelated to endothelial factors but is associated with interference with the Ca^{2+} supply, with greater effect on intracellular Ca^{2+}-dependent contractions as well as activation of glibenclamide-sensitive K^+ channels. Interestingly, the action of cicletanine was more pronounced on arteries from patients with pregnancy-induced hypertension.

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Capsaicin-sensitive vagal stimulation-induced gastric acid secretion in the rat: evidence for cholinergic vagal afferents

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1 The effects of electrical vagal stimulation on frequency-dependent gastric acid secretion were investigated in urethane-anaesthetized rats *in vivo*.

2 Stimulation at 4, 16 or 32 Hz was performed in rats treated with atropine ($1\,mg\,kg^{-1}$, i.v.), hexamethonium ($10\,mg\,kg^{-1}$, i.v. bolus and $1\,mg\,kg^{-1}\,min^{-1}$, i.v. infusion) or atropine and hexamethonium (doses as above); in some experiments pentagastrin ($1.2\,\mu g\,kg^{-1}\,h^{-1}$, i.v. infusion) was infused prior to stimulation.

3 Maximal acid secretion occurred at 16 Hz. This was significantly reduced but not abolished by atropine or hexamethonium and completely abolished after atropine and hexamethonium. In the presence of pentagastrin, the acid secretory response to 16 Hz stimulation was augmented, atropine or hexamethonium reduced stimulated secretion by about 70%, whereas atropine and hexamethonium completely abolished stimulated secretion.

4 In rats in which the vagus nerve was pretreated with capsaicin 10–14 days before experimentation there was a significant reduction (by about 40%) in stimulated acid secretion at 16 Hz, which was virtually abolished by atropine treatment. After acute treatment of the vagus nerve with capsaicin (at the time of experimentation) maximally stimulated acid secretion was significantly reduced by about 50%.

5 Taken together, these results indicate that capsaicin-sensitive afferent fibres contribute to the acid secretory response induced by electrical vagal stimulation in the rat. Based on pharmacological evidence, the capsaicin-sensitive afferent fibres may be cholinergic, since atropine and hexamethonium totally abolish vagal stimulation-induced acid secretion.

Keywords: gastric acid secretion; capsaicin; vagus nerve; atropine; hexamethonium

Introduction

Capsaicin has been widely used to study the structure and function of primary afferent neurones, since it was discovered that it induced the selective degeneration of a population of chemosensitive primary sensory nerves (Jancsó *et al.*, 1977) and see reviews by Buck & Burks (1986), Holzer (1988) and Maggi & Meli (1988). A number of studies have focused on the role of capsaicin-sensitive nerves in the stomach. A population of capsaicin-sensitive nerves, of spinal afferent origin, have been described in the gastric muscle and mucosa (Sharkey *et al.*, 1984; Sternini *et al.*, 1987; Su *et al.*, 1987; Green & Dockray, 1988). Ablation of primary afferent neurones by capsaicin treatment augmented mucosal damage produced by acid distension (Szolcsanyi & Bartho, 1981), pyloric ligation (Szolcsanyi & Bartho, 1981), indomethacin (Evangelista *et al.*, 1986; Holzer & Sametz, 1986), ethanol (Holzer & Sametz, 1986; Esplugues & Whittle, 1990) or platelet-activating factor (PAF) (Esplugues *et al.*, 1989) but was ineffective in augmenting ulcer formation produced by cold-restraint (Dugani & Glavin, 1986).

The effects of capsaicin on gastric acid secretion are unclear. Oral administration of capsaicin increased acid secretion in a dose-dependent manner (Limlomwongse *et al.*, 1979). Adult rats treated with capsaicin systemically ($300\,mg\,kg^{-1}$, s.c.; Alföldi *et al.*, 1986) or topically (on to the vagus, 1 mg; Raybould & Taché, 1989; Raybould *et al.*, 1990) had attenuated secretory responses to histamine (i.v.) and a thyrotropin releasing hormone (TRH) analogue (intracisternally), but not to pentagastrin, bethanechol or carbachol (i.v.). In contrast, adult rats treated with a low dose of capsaicin systemically ($65\,mg\,kg^{-1}$, s.c.) had a depressed response to pentagastrin (other secretagogues were not tested) (Dugani & Glavin, 1986). Finally, animals treated at birth with capsaicin showed no impairment in their responsiveness to any gastric secretagogue (Esplugues *et al.*, 1990).

In the rat, a population of capsaicin-sensitive vagal afferent fibres have been described (Gamse *et al.*, 1981; Marsh *et al.*, 1987; Waddell & Lawson, 1989) and it has been demonstrated that these nerves partially mediate the increase in acid secretion in response to gastric distension (Raybould & Taché, 1989; Esplugues *et al.*, 1990). It is also well established that stimulation of the vagus nerve gives rise to atropine- and/or hexamethonium-resistant excitatory motor responses in the stomach (Delbro *et al.*, 1982; Fandriks & Delbro, 1983; Yano *et al.*, 1983; Okamoto *et al.*, 1986; Morishita & Guth, 1986; Beck *et al.*, 1988; Tsubomura *et al.*, 1987; 1988). Some of these effects have been attributed to the activation of unmyelinated vagal afferent fibres (Delbro *et al.*, 1982; Fandriks & Delbro, 1983; Tsubomura *et al.*, 1988). However, the potential contribution that capsaicin-sensitive vagal afferent fibres make to acid secretion induced by electrical stimulation is not known.

Hence in the present study we have investigated the effects of electrical vagal stimulation on frequency-dependent acid secretion in the presence and absence of atropine and/or hexamethonium and after perineural application of capsaicin on the vagus nerve in the anaesthetized rat. A preliminary account of this work has been published (Sharkey *et al.*, 1990).

Methods

Animals

Adult male Sprague-Dawley rats (Charles River) with a body weight of 312 ± 6 g (mean ± s.e.mean) were assigned to one of two groups to receive capsaicin or vehicle treatment 7–14 days before experimentation (pretreated group, $n = 16$) or topical application of capsaicin, vehicle or paraffin oil at the time of experimentation (acute group, $n = 53$).

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Capsaicin treatment

Pretreatment Animals were anaesthetized with Halothane (Fluothane, Ayerst; induced with 4% and maintained on 2%) in oxygen. Under aseptic conditions the left cervical vagus nerve was isolated with the aid of a dissecting microscope. A strip of parafilm (Fisher) was placed under the nerve and two small cotton balls were placed on either side of the nerve to prevent leakage of the capsaicin or vehicle over the surrounding tissues. Capsaicin (Sigma; 10 mg ml^{-1}, dissolved in 10% Tween 80 and 90% paraffin oil [vehicle], $n = 10$) or vehicle ($n = 6$) was applied to a 3–5 mm section of nerve for 30 min (approximate dose 1 mg/rat), after which the area was cleaned and thoroughly rinsed with sterile 0.9% saline (Jancsó & Such, 1983; Raybould & Taché, 1989). All animals received a single prophylactic injection of penicillin G (15,000 units, i.m.). The incision was closed and the animals were allowed to recover for 7–14 days before experimentation.

Acute treatment The acutely-treated group contained animals receiving (a) capsaicin ($n = 9$, dissolved as above), (b) vehicle alone ($n = 9$) or (c) paraffin oil alone ($n = 35$). The capsaicin or vehicle was applied to the nerve at the site of the stimulating electrode as described above.

Measurement of gastric acid secretion

Animals were prepared according to a modification of the method of Ghosh & Schild (1958). Rats were anaesthetized with urethane (1.5 g kg^{-1}, i.p.; Sigma), placed on a heating pad and maintained at 35° ± 1°C. A 3–5 mm section of the left cervical vagus nerve was isolated, the central end was cut or, in some experiments (acute capsaicin treatment) crushed and ligated and the peripheral portion was placed on a bipolar silver stimulating electrode and immersed in paraffin oil. The stomach was perfused through an orogastric tube (3 mm o.d.) with 0.9% sodium chloride at 37°C at rate of 1.3 ml min^{-1} by use of a roller pump (LKB). Effluent from the stomach was collected by a tube introduced through the pylorus and connected directly to a Radiometer Copenhagen Automatic Titration system enabling continuous monitoring of gastric acid output. The end point of all titrations was pre-set to the pH of the perfusate (about pH 6.5) the titrant used was 0.01 M sodium hydroxide calibrated daily with 0.01 M hydrochloric acid standard.

Blood pressure was monitored from a femoral artery and used to check (a) the anaesthetic level of the animal, (b) the efficacy of the nerve stimulations (by observing bradycardia) and (c) the action of autonomic antagonists given to the animal. Drugs were administered intravenously through a femoral venous cannula which was also used for fluid replacement. Vagal stimulations (1 ms pulse, 4–32 Hz, 12–15 V) were delivered for 1 min from a Grass S48 stimulator connected to a stimulus isolator (Grass Instruments).

Drugs

The following drugs were used: atropine sulphate (Sigma; 1 mg kg^{-1}, i.v. bolus), hexamethonium bromide (Sigma; 10 mg kg^{-1}, i.v. bolus and 1 mg kg^{-1} min^{-1}, i.v. infusion) and pentagastrin (Peptavlon, Ayerst Laboratories, 1.2 μg kg^{-1} h^{-1}, i.v. infusion). All drugs were dissolved in 0.9% saline.

Experimental protocol

Animals were allowed to stabilize gastric acid output for a period of 30–45 min. When at least 10 min of stable acid secretion (μmol H$^+$ 5 min^{-1}) was measured (basal acid output) the vagus nerve was stimulated. Acid secretion was continuously monitored until it returned to basal levels, after which additional stimulations were performed in a random fashion. If basal secretion before and after stimulation was not the same, the mean of the pre- and post-stimulation basal values (defined as 10 min of stable secretion) were calculated and used as 'basal'. This procedure was repeated in the presence or absence of autonomic antagonists and in some experiments after pentagastrin infusion.

In one set of experiments the acute effects of capsaicin were studied. In these experiments the vagus nerve was stimulated in paraffin oil, vehicle and capsaicin (dissolved as above). The capsaicin was left on the nerve for 30 min, after which the nerve was put back in paraffin oil and re-stimulated.

Data analysis

The response to each stimulation was calculated as total acid output with basal subtracted for the period of the response. Data from capsaicin-treated or control animals were compared by analysis of variance for repeated measures with Newman-Keuls test for multiple comparisons, paired or unpaired Student's t tests as applicable. Statistical values reaching probabilities of $P < 0.05$ or less were considered significant.

Results

Basal acid secretion

The mean basal acid output in control (untreated) rats was 1.8 ± 0.2 μmol H$^+$ 5 min^{-1} (mean ± s.e.mean, $n = 39$ rats), in vehicle-treated rats was 1.8 ± 0.1 μmol H$^+$ 5 min^{-1} ($n = 10$) and in capsaicin pretreated rats was 1.8 ± 0.3 μmol H$^+$ 5 min^{-1} ($n = 13$).

Responses to electrical stimulation: untreated controls

Left cervical vagal stimulation in control (untreated) rats gave a graded increase in gastric acid secretion that peaked at a stimulation frequency of 16 Hz (Figures 1 and 2). The response at 32 Hz was lower than at 16 Hz ($P < 0.05$, ANOVA) and both 16 Hz and 32 Hz gave greater responses than 4 Hz stimulation. The maximum acid output (basal subtracted) to 16 Hz stimulation in control rats was 10.7 ± 1.0 μmol H$^+$ ($n = 17$). The time course of stimulation was similar at all frequencies examined; acid output increased 5 min after stimulation,

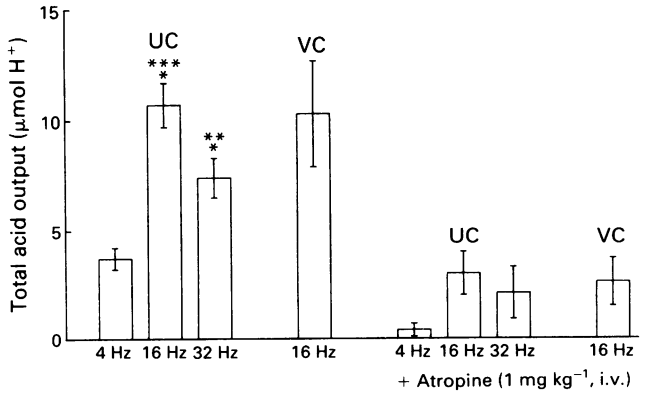

Figure 1 Effects of vagal stimulation at 4, 16 and 32 Hz on total gastric acid output (basal subtracted) from control rats in the presence or absence of atropine (1 mg kg^{-1}, i.v.). UC, untreated controls, VC, vehicle-treated controls (see text for details). *Significantly >4 Hz controls, $P < 0.05$ ANOVA; **significantly <16 Hz controls, $P < 0.05$ ANOVA; ***significantly >16 Hz capsaicin-treated rats (see Figure 3), $P < 0.05$ ANOVA. Atropine significantly reduced acid secretion at all frequencies tested.

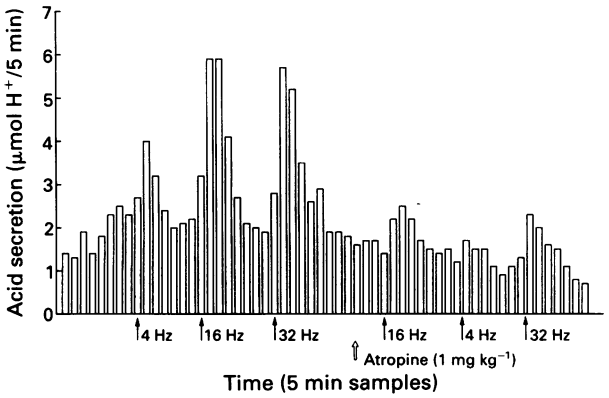

Figure 2 Stimulation of acid secretion in a single experiment from a control rat in the presence and absence of atropine (1 mg kg^{-1}, i.v.). Note that basal output is not subtracted. Each column represents acid output over 5 min. Stimulation was for 1 min at 4, 16 and 32 Hz (supramaximal voltage, 1 ms pulse).

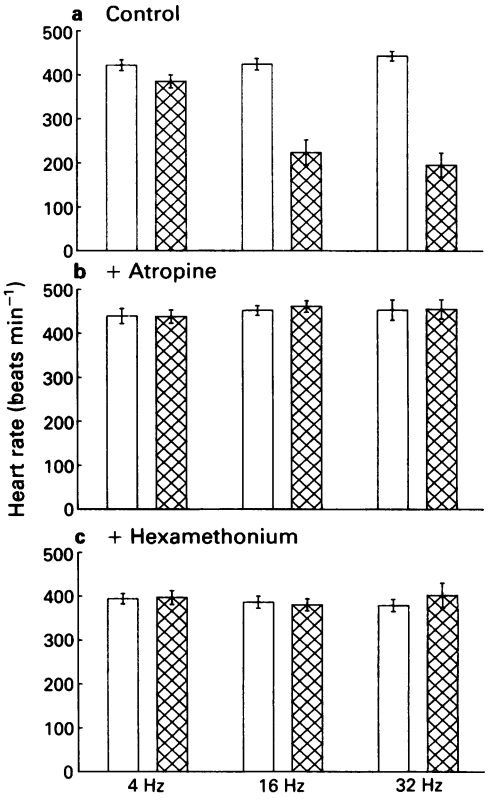

Figure 3 The effects of vagal stimulation at 4, 16 and 32 Hz on heart rate in normal rats (a) (4 Hz, $n = 13$; 16 Hz, $n = 16$; 32 Hz, $n = 14$), rats treated with atropine (b) (1 mg kg^{-1}, i.v.; 4 Hz, $n = 7$; 16 Hz, $n = 11$; 32 Hz, $n = 5$) or rats treated with hexamethonium (c) (10 mg kg^{-1}, i.v. bolus and 1 mg kg^{-1} min^{-1}, i.v. infusion; 4 Hz, $n = 3$; 16 Hz, $n = 6$; 32 Hz, $n = 4$). Open columns: unstimulated; cross-hatched columns: stimulated. Resting heart rate was lowered by hexamethonium (ANOVA, $P < 0.05$) and slightly raised by atropine. Note that bradycardia was abolished after treatment with either antagonist.

peaked at 10–15 min and returned to basal conditions within 30–40 min of stimulation (Figure 2). The frequency-response data is shown in Figures 1 and 2 and Table 1. During stimulation bradycardia was observed in all animals and in some animals this was quantified and is shown in Figure 3.

In the presence of atropine, there was a reduction, though not abolition, of the response to electrical stimulation in control animals (Figures 1 and 2 and Table 1). At 16 Hz, atropine reduced acid secretion by about 70%, whereas at 4 Hz there was an almost total abolition of the response to stimulation. Bradycardia was not observed in animals treated with atropine (Figure 3). Mean arterial blood pressure in the atropine-treated rats was lower than in control rats before atropine treatment (75 ± 3 mmHg compared to 85 ± 4 mmHg, $P < 0.01$, paired t test).

In the presence of a dose of hexamethonium sufficient to block all cardiac effects of electrical vagal stimulation (Figure 3), stimulated acid secretion was not completely abolished in control rats (Table 1). At 16 Hz there was a 90% reduction in acid output. However, mean arterial blood pressure was reduced in hexamethonium-treated rats (49 ± 4 mmHg compared to 85 ± 4 mmHg, $P < 0.01$, paired t test). Combination of hexamethonium and atropine completely blocked all cardiac effects of electrical vagal stimulation and gastric acid secretion. The mean arterial blood pressure in these rats was also lower than in controls (38 ± 3 mmHg, $P < 0.01$, paired t test).

In the presence of a continuous infusion of pentagastrin (1.2 μg kg^{-1} h^{-1}) acid output was approximately doubled in control rats from about 1.8 to 3.3 ± 0.6 μmol H$^+$ 5 min^{-1} ($n = 18$). Stimulation of the vagus nerve at 16 Hz in these

Table 1 Gastric acid secretion in rats in response to electrical stimulation: effects of atropine, hexamethonium, pentagastrin and capsaicin

Stimulation frequency		*+ Atropine*	*+ Hexamethonium*	*+ Atropine + hexamethonium*
Untreated controls				
4 Hz	3.7 ± 0.5 (17)	0.4 ± 0.3 (5)	0.4 ± 0.2 (5)	0 (4)
16 Hz	10.7 ± 1.0 (17)*.**.***	3.0 ± 1.0 (6)	1.1 ± 0.3 (6)	0 (5)
32 Hz	7.4 ± 0.9 (17)*	2.1 ± 1.2 (3)	0.8 ± 0.3 (6)	0 (5)
+ Pentagastrin				
16 Hz	19.1 ± 3.0 (18)†	5.6 ± 1.8 (6)	5.0 ± 1.6 (7)‡	0.4 ± 0.3 (5)
Capsaicin pretreated				
4 Hz	3.6 ± 1.2 (9)	0 (2)	0 (3)	
16 Hz	6.3 ± 1.1 (10)*	0.6 ± 0.3 (4)	0.5 (2)	
32 Hz	5.6 ± 1.2 (10)*	0.3 ± 0.3 (3)	0 (3)	

Values (μmol H$^+$) are: mean ± s.e.mean (n).
* Significantly > 4 Hz within group (ANOVA, $P < 0.05$); **significantly > 32 Hz within group (ANOVA, $P < 0.05$); ***significantly > 16 Hz compared to capsaicin-treated group (ANOVA, $P < 0.05$).
† Significantly > 16 Hz between all groups (t test, $P < 0.01$).
‡ Significantly > untreated controls (t test, $P < 0.01$).
Note that atropine and hexamethonium significantly reduced acid secretion at all frequences in all groups.

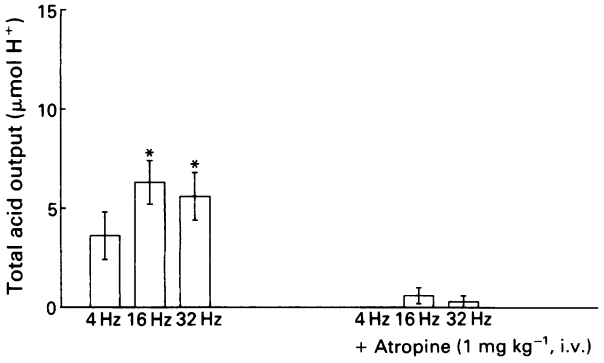

Figure 4 Effects of vagal stimulation at 4, 16 and 32 Hz on total gastric acid output (basal subtracted) from capsaicin-treated rats in the presence or absence of atropine (1 mg kg^{-1}, i.v., see text for details). *Significantly >4 Hz controls, $P < 0.05$ ANOVA. Atropine significantly reduced acid secretion at all frequencies tested.

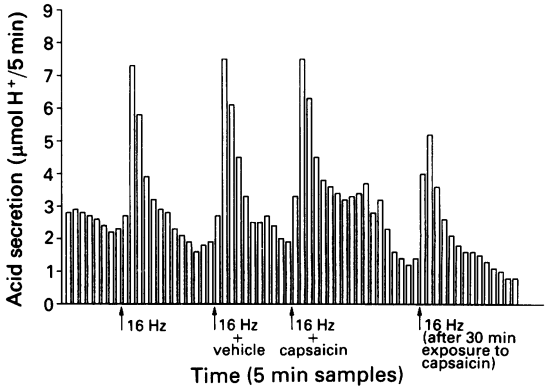

Figure 7 Results of an individual experiment in which vagal stimulation at 16 Hz was performed in the presence of paraffin oil (unlabelled), vehicle (10% Tween 80, 90% paraffin oil) or capsaicin (10 mg ml^{-1} dissolved in vehicle), see text for details. Note that basal secretion is not subtracted. Also that this animal had an elevated basal secretion at the start of the experiment which 'ran down' during the experiment.

animals gave an augmented acid response (Table 1), the total acid output was 19.1 ± 3.0 μmol H$^+$ 5 min^{-1}, which was greater than for untreated control rats ($P < 0.05$, t test, $n = 18$). In the presence of atropine, acid output was reduced by 70% as before, however, the response in the presence of hexamethonium was also only reduced by 70% (cf. 90% in the previous experiment, see Table 1) in the face of the reduced blood pressure. Combination of atropine and hexamethonium completely abolished the response to vagal stimulation in the presence of pentagastrin.

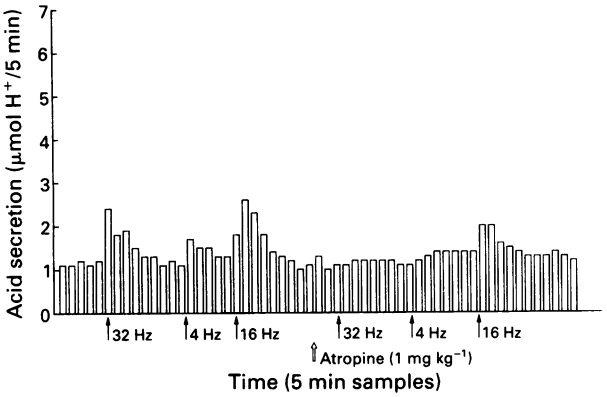

Figure 5 Simulation of acid secretion in a single experiment from a capsaicin-treated rat in the presence and absence of atropine (1 mg kg^{-1}, i.v.). Note that basal output is not subtracted. Each column represents acid output over 5 min. Stimulation was for 1 min at 4, 16 and 32 Hz (supramaximal voltage, 1 ms pulse).

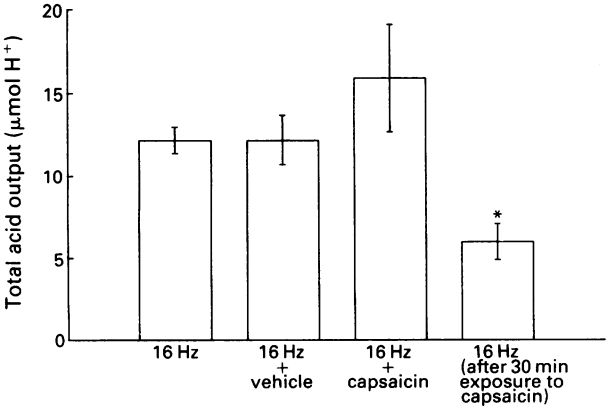

Figure 6 Effects of vagal stimulation at 16 Hz in the presence of paraffin oil (unlabelled), vehicle (10% Tween 80, 90% paraffin oil) or capsaicin (10 mg ml^{-1} dissolved in vehicle), see text for details. *Significantly <control, $P < 0.01$ paired t test.

Responses to electrical stimulation: vehicle-treated controls

Rats pretreated with the vehicle used to dissolve the capsaicin showed no significantly different responses from that of untreated control rats (Figure 1). Stimulation of the vagus caused bradycardia in these animals.

Responses to electrical stimulation

Capsaicin-pretreated animals Perineural application of capsaicin 7–14 days before vagal stimulation caused a reduction in the response at 16 Hz compared to controls ($P < 0.05$, ANOVA), a non-significant reduction at 32 Hz but almost no effect on the response at 4 Hz (Figures 4 and 5 and Table 1). Furthermore, capsaicin-treatment virtually abolished the differences between responses at the three different frequencies of stimulation. In the presence of either atropine or hexamethonium, the response to vagal stimulation was almost completely abolished at all frequencies (Table 1). Bradycardia was again observed in all animals during stimulation prior to treatment with an autonomic blocker.

Acute capsaicin treatment In this group of rats, stimulation of the left cervical vagus at 16 Hz gave a total acid output of 12.2 ± 0.8 μmol H$^+$ ($n = 8$), in the presence of the vehicle the response was 12.2 ± 1.5 μmol H$^+$ ($n = 5$) (Figures 6 and 7). Electrical stimulation during capsaicin treatment gave a slightly augmented response of 15.9 ± 3.2 μmol H$^+$ ($n = 6$), however, after 30 min of exposure to capsaicin the response significantly declined to 6.0 ± 1.1 μmol H$^+$ ($n = 8$, $P < 0.01$, paired t test). At all rates of stimulation bradycardia was again observed and in 2 animals it was quantified. The pre-stimulation heart rate was 430 beats per min, which was reduced to 147 beats per min by stimulation at 16 Hz in the presence of vehicle. In the presence of capsaicin, stimulation-induced bradycardia was 141 beats per min and stimulation after capsaicin was 175 beats per min. At the end of the

experiment the heart rate was unchanged at 430 beats per min.

Discussion

We have demonstrated that capsaicin-sensitive fibres in the vagus nerve contribute to vagal stimulation-induced gastric acid secretion in the anaesthetized rat *in vivo*. We also demonstrated that under appropriate conditions there is an atropine- or hexamethonium-resistant component of acid secretion.

Direct application of capsaicin to the vagus nerve leads to a prolonged and selective blockade in sensitive afferent fibres. Use of this allowed Raybould & Taché (1989) to demonstrate that capsaicin-sensitive vagal fibres partially mediate the acid secretory response to distension. These studies have been extended by Esplugues *et al.* (1990) who showed that local application of capsaicin to the coeliac ganglion also decreased the secretory response to gastric distension. In this context Esplugues *et al.* (1989) and Esplugues & Whittle (1990) also demonstrated that morphine or its analogues augment gastric damage induced by ethanol or PAF, through a mechanism that involves capsaicin-sensitive afferent fibres. In the present study, capsaicin was applied to the vagus under acute or chronic experimental paradigms. In both cases a similar result was observed; a reduction in maximal acid secretion by about 40 to 50%. Stimulation at low frequencies (4 Hz) produced comparable responses in control and capsaicin-treated rats, suggesting that fibres activated by high frequency stimulation are more susceptible to capsaicin. Because of the specificity of capsaicin this may be interpreted as preferential activation of efferent fibres at low frequencies of stimulation and afferent fibres at high frequencies.

The effects of electrical vagal stimulation at varying frequencies on gastric acid output have previously been investigated in the rat (Berthoud *et al.*, 1986), cat (Martinson, 1965) and ferret (Grundy & Scratcherd, 1982). In the rat, Berthoud *et al.* (1986) obtained a frequency-response curve similar to that found in the present study, with the reduction in secretion at higher stimulation frequencies, presumably due to conduction block. In that study the peak occurred at about 4 Hz, whereas in our study acid output peaked around 16 Hz. This difference may be due to different stimulation systems; Berthoud *et al.* (1986) used constant current stimulation whilst constant voltage was used in this study.

In this study we demonstrated an apparent atropine-resistant component of vagal stimulation-induced acid secretion. This has been observed previously in the rat (Stapelfeldt *et al.*, 1988), as has a vagally-stimulated atropine-resistant gastric vasodilatation (Yano *et al.*, 1983). The dose of atropine given ($1\,mg\,kg^{-1}$) was sufficient to block the cardiovascular effects of vagal stimulation, however, higher doses of atropine were not examined. That combined cholinergic blockade (atropine and hexamethonium) blocked acid secretion suggested that cholinergic nerves may be responsible for mediating vagal stimulation-induced acid secretion. The potential contribution of adrenergic mechanisms was not examined but Stapelfeldt *et al.* (1988) demonstrated a small adrenergic component in vagally-stimulated acid secretion. They also demonstrated that naloxone augmented acid secretion suggesting that endogenous opiates normally have an inhibitory action on vagally-mediated secretion.

The observation that cholinergic nerves are largely responsible for vagally-stimulated acid secretion is further supported by the results of stimulation in the presence of pentagastrin. In this case even when acid secretion was augmented, combined cholinergic blockade abolished stimulated acid secretion. The observation that pentagastrin potentiated the effects of vagal stimulation has been described previously and is reviewed by Debas (1987). The effects of hexamethonium on acid secretion are complicated by its profound hypotensive action. However, when hexamethonium was given in the presence of pentagastrin, even though blood pressure was significantly reduced, secretion was still maintained and was, in fact, comparable to that seen after atropine treatment.

Taken together, these results suggest that the capsaicin-sensitive component of vagal stimulation-induced gastric acid secretion is mediated by cholinergic neurones. One possible interpretation of these data is that vagal afferents are themselves cholinergic. There is a substantial body of evidence that demonstrates a sub-population of vagal afferent neurones in the rat (Helke *et al.*, 1983; Palouzier *et al.*, 1987; Ternaux *et al.*, 1989) and cat (Fujiwara & Kurahashi, 1976; Fujiwara *et al.*, 1978) are cholinergic and some evidence for gastric cholinergic vagal afferents (Tsubomura *et al.*, 1987; 1988). It is interesting to note that of the neuropeptides found in the nodose ganglia (notably substance P and calcitonin gene-related peptide) only a very small proportion were found to project to the stomach (Dockray & Sharkey, 1986; Green & Dockray, 1988). Thus, electrical vagal stimulation may result in activation of efferent preganglionic fibres and afferent fibres both of which are cholinergic. Recently, Tayo and coworkers (Tayo & Williams, 1988; Tayo & Helke, 1990) have provided strong evidence that some gastric vagal motor neurones are apparently non-cholinergic and may contain dopamine. However, we found no functional evidence for inhibitory actions of vagal stimulation in the rat *in vivo*, as has been described in the mouse stomach *in vitro* (Davison & Najafi-Farashah, 1987). Thus the simplest model that explains the data in this study is that the hexamethonium-resistant component of stimulated acid secretion is due to capsaicin-sensitive afferent fibres, which are atropine-sensitive. The atropine-resistant component may be partially afferent (non-cholinergic) or more likely may represent a vagal efferent non-cholinergic response. Since a vagal non-cholinergic gastrin secretion has already been described (Debas, 1987; Walsh, 1989) it is conceivable that this contributes to the atropine-resistant component of acid secretion.

In conclusion, these results indicate that capsaicin-sensitive afferent fibres contribute to the acid secretory response induced by electrical vagal stimulation in the rat. Based on pharmacological evidence, capsaicin-sensitive afferent fibres give rise to cholinergic actions, since atropine and hexamethonium (at the doses used in this study) totally abolished vagal stimulation-induced acid secretion. This observation is consistent with that of Limlomwongse *et al.* (1979) who demonstrated that intragastric capsaicin-stimulated acid secretion could be blocked by cholinoceptor antagonists. At this time it is not clear if cholinergic neurones are the final mediator of the acid secretory response or if they act to release another agent (e.g. a peptide). The physiological significance of cholinergic vagal afferent fibres remains to be elucidated, but its interesting to note that acute capsaicin administration was found to be protective in ulcer formation (Szolcsanyi & Bartho, 1981; Holzer & Lippe, 1988; Holzer *et al.*, 1989; 1990). This suggests that capsaicin-sensitive vagal afferents in the stomach may play a role in protective reflexes in that organ.

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Possible dependence of pressor and heart rate effects of N^G-nitro-L-arginine on autonomic nerve activity

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1 The effects of N^G-nitro-L-arginine (L-NNA) on mean arterial pressure (MAP) and heart rate (HR) were investigated in conscious rats.

2 Intravenous bolus cumulative doses of L-NNA (1–32 mg kg^{-1}) dose-dependently increased MAP. Both mecamylamine and phentolamine increased MAP responses to L-NNA, angiotensin II and methoxamine. Propranolol, reserpine, atropine and captopril did not affect MAP response to L-NNA.

3 A significant negative correlation of HR and MAP responses to L-NNA was obtained in control rats but not in rats pretreated with reserpine or mecamylamine. Significant negative correlations also occurred in the presence of atropine, propranolol, phentolamine or captopril.

4 A single i.v. bolus dose of L-NNA (32 mg kg^{-1}) raised MAP to a peak value of 53 ± 3 mmHg and the effect lasted more than 2 h; the rise and recovery of MAP were accompanied by significant decrease and increase in HR, respectively. While both phentolamine and mecamylamine increased peak MAP response to L-NNA, mecamylamine abolished the biphasic HR response and phentolamine potentiated the bradycardic component of HR.

5 Blockade of the autonomic nervous and renin-angiotensin systems did not attenuate the pressor effects of L-NNA. However, the biphasic HR response to L-NNA is mediated via modulation of autonomic nerve activities.

Keywords: N^G-nitro-L-arginine (L-NNA); vasopressor; autonomic ganglion; sympathetic and parasympathetic nervous system; renin-angiotensin system

Introduction

There is evidence that endothelium-derived relaxing factor (EDRF) released by vascular endothelial cells is nitric oxide (NO) (Palmer *et al.*, 1987; Ignarro *et al.*, 1987) which is formed from the precursor L-arginine (L-Arg) (Palmer *et al.*, 1988; Sakuma *et al.*, 1988). It has been shown that NO synthase and endothelium-dependent vascular relaxation responses in isolated arteries are inhibited by N^G-substituted L-Arg analogues which include N^G-monomethyl-L-arginine (L-MMA) (Palmer *et al.*, 1988; Rees *et al.*, 1989a; 1990), N^G-nitro-L-arginine methyl ester (Moore *et al.*, 1990; Rees *et al.*, 1990), N-iminoethyl-L-ornithine (Rees *et al.*, 1990) and N^G-nitro-L-arginine (L-NNA) (Moore *et al.*, 1990; Mülsch & Busse, 1990; Kobayashi & Hattori, 1990; Ishii *et al.*, 1990). *In vivo* studies show that L-MMA (Rees *et al.*, 1989b; 1990; Aisaka *et al.*, 1989; Whittle *et al.*, 1989; Gardiner *et al.*, 1990b,c), N^G-nitro-L-arginine methyl ester (Gardiner *et al.*, 1990a,c,d), N-iminoethyl-L-ornithine (Rees *et al.*, 1990) and L-NNA (Wang & Pang, 1990) cause pressor responses and bradycardia.

Although it is likely that the pressor effects of L-Arg analogues are caused by the inhibition of NO production from vascular endothelial cells (Aisaka *et al.*, 1989; Rees *et al.*, 1989b; Wang & Pang, 1990), other vasopressor systems may contribute to the response. It has been shown that vascular endothelium inhibits the release of noradrenaline from sympathetic nerves which innervate canine pulmonary artery and vein suggesting that the endothelium, in part via endothelium-derived relaxing factor (EDRF) release, acted as an endogenous inhibitor of sympathetic transmitter release (Greenberg *et al.*, 1990). Togashi *et al.* (1990) showed that L-MMA increased postganglionic sympathetic nerve activities in intact and bilateral sino-aortic- and vagal-denervated rats and preganglionic adrenal nerve activity in sino-aortic and vagal-denervated rats. It has also been reported that EDRF inhibited renin release (Vidal *et al.*, 1988). Therefore it is logical to postulate that the haemodynamic effects of L-Arg analogues are partially mediated via potentiation of activities of the autonomic nervous and/or renin-angiotensin system(s).

The aims of this study were: (1) to assess the contribution of the autonomic nervous and renin-angiotensin systems on pressor response to L-NNA; (2) to examine whether the bradycardia produced in response to L-NNA is mediated via reflex activation of the autonomic nervous system.

Methods

Surgical preparation

Sprague-Dawley rats (240–400 g) were anaesthetized with halothane (4% in air for induction, 2% in air for surgical preparation). A polyethylene cannula (PE50) was inserted into the left iliac artery to allow recordings of mean arterial pressure (MAP). PE50 cannulae were also inserted into the right (or both) iliac vein(s) for the administration of drugs. The cannulae were filled with heparinized saline (25 iu ml^{-1}) and tunnelled s.c. along the back, exteriorized at the back of the neck and secured. The rats were given 6–7 h recovery of the effects of halothane and surgery before further use.

Experimental protocol

The indwelling arterial catheter from each rat was connected to a pressure transducer (P23DB, Gould Statham, CA, U.S.A.) for the recordings of MAP and heart rate (HR) which was derived electronically from the upstroke of the arterial pulse pressure by a tachograph (Grass, Model 7P4G). The conscious rats were allowed to wander freely in a small cage for 1 h before the administrations of drugs. MAP and HR were continuously monitored. The rats were killed by an overdose of pentobarbitone at the end of each experiment. Two main studies were conducted:

Dose-response curves for N^G-nitro-L-arginine Rats, randomly divided into seven groups ($n = 6$ each), were pretreated with: (I) normal saline (0.9% NaCl); (II) phentolamine (i.v. infusion,

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Table 1 Baseline values of mean arterial pressure (MAP) and heart rate (HR) (mean ± s.e.mean) in conscious rats prior to and 40 min after the administration of normal saline, phentolamine, propranolol, reserpine, mecamylamine, atropine and captopril

	MAP (mmHg)		*HR* (beats min^{-1})	
Antagonists	*Before*	*After*	*Before*	*After*
Protocol 1				
Normal saline	103 ± 5	104 ± 5	403 ± 10	410 ± 8
Phentolamine	106 ± 4	69 ± 7*	442 ± 12	518 ± 18*
Propranolol	104 ± 4	104 ± 4	364 ± 10	334 ± 8*
Reserpine	—	80 ± 5†	—	295 ± 11†
Mecamylamine	113 ± 3	88 ± 4*	377 ± 9	313 ± 13*
Atropine	116 ± 2	118 ± 3	370 ± 23	432 ± 18*
Captopril	105 ± 6	103 ± 5	372 ± 19	418 ± 16*
Protocol 2				
Normal saline	106 ± 3	104 ± 3	343 ± 13	344 ± 13
Mecamylamine	108 ± 4	69 ± 2*	431 ± 10	308 ± 8*
Phentolamine	101 ± 5	72 ± 3*	401 ± 13	443 ± 8*

* Denotes significant difference from corresponding control values within the same group ($P < 0.05$); † Denotes significant difference from normal saline group ($P < 0.05$). $n = 6$ per group.

300 μg kg^{-1} min^{-1}); (III) propranolol (i.v. bolus at 1 mg kg^{-1} followed by infusion at 1.6 μg kg^{-1} min^{-1}); (IV) reserpine (5 mg kg^{-1}, i.p., 24 h prior to the study); (V) mecamylamine (i.v. bolus at 10 mg kg^{-1} followed by infusion at 300 μg kg^{-1} min^{-1}); (VI) atropine (i.v. bolus at 10 mg kg^{-1} followed by infusion at 8 μg kg^{-1} min^{-1}) and (VII) captopril (20 mg kg^{-1}, i.v. bolus). With the exception of reserpine and captopril, all antagonists were continuously infused for approximately 160 min, i.e., to the end of the experiment. Cumulative doses of L-NNA (1–32 mg kg^{-1}, i.v. bolus) were given 40 min after the administration of the vehicle or blockers at dose-intervals of 15–20 min, the period required to obtain steady state MAP responses. A single dose of angiotensin I (1 μg kg^{-1}), methoxamine (20 or 30 μg kg^{-1}), acetylcholine (1 μg kg^{-1}) or isoprenaline (1 μg kg^{-1}) was injected as an i.v. bolus prior to and 20 min after the start of administration of captopril, phentolamine, atropine or propranolol, respectively, and again 2 h after giving L-NNA to assess the degrees of inhibition at the start and completion of the studies. In rats pretreated with reserpine and vehicle, tyramine (200 μg kg^{-1}) was injected as an i.v. bolus 20 min prior to and 2 h after giving L-NNA. Excluding the equilibration time, the duration of each study was approximately 3 h.

Time course of responses to a single dose of N^G-nitro-L-arginine Another three groups of rats ($n = 6$ each) were pretreated with: (VIII) normal saline; (IX) mecamylamine; (X) phentolamine, at the same doses as those described previously. In phentolamine and mecamylamine groups, angiotensin II was injected as an i.v. bolus prior to and 20 min after the administration of a blocker. In Groups VIII, IX and X, a single dose of L-NNA (32 mg kg^{-1}) was injected as an i.v. bolus 40 min after the start of administration of a blocker. MAP and HR were continuously monitored for 2 h.

Drugs

The following drugs were obtained from Sigma Chemical Co. (MO, U.S.A.): N^G-nitro-L-arginine (L-NNA), mecamylamine hydrochloride, atropine sulphate, Des-Asp^1-angiotensin I acetate, angiotensin II acetate, acetylcholine hydrochloride, (±)-propranolol hydrochloride, (−)-isopropylnoradrenaline hydrochloride and tyramine hydrochloride. The following drugs were also used: phentolamine hydrochloride (Ciba Pharmaceutical Co., NJ, U.S.A.), methoxamine hydrochloride (B.W. & Co. Ltd., Quebec, Canada), captopril (E.R. Squibb & Sons Inc., NJ, U.S.A.) and reserpine (Ciba Pharmaceutical Co., Quebec, Canada). All drugs were dissolved in normal saline.

Calculation and statistical analysis

The ED_{50} and maximum response (E_{max}) values of L-NNA were obtained from individual dose-response curves. Correlation coefficient (r), slope and intercept were calculated from individual HR versus MAP curves at various doses of L-NNA. Rise phase $t_{1/2}$ of L-NNA were obtained from time-course curves. To obtain normal distribution of rise phase $t_{1/2}$, the data were logarithmically-transformed prior to statistical analysis. All data were analyzed by the analysis of variance followed by Duncan's multiple range test with $P < 0.05$ selected as the criterion for statistical significance. All results are expressed as mean ± standard error (s.e.mean) except for rise phase $t_{1/2}$ which is expressed as geometric mean and 95% confidence range.

Results

Effects of antagonists on mean arterial pressure and heart rate

Table 1 shows baseline MAP and HR (protocol 1: Groups I to VII; protocol 2: Groups VIII to X) in rats prior to and 40 min after pretreatment with normal saline, phentolamine, propranolol, reserpine, mecamylamine, atropine or captopril. Normal saline (protocols 1 and 2) affected neither MAP nor HR. MAP was not affected by propranolol, atropine or captopril but significantly decreased by phentolamine, reserpine and mecamylamine. HR was decreased by reserpine, mecamylamine and propranolol and increased by phentolamine, atropine and captopril.

Table 2 shows the effects of the antagonists on responses to several agonists. Phentolamine (Group II) completely blocked

Table 2 The effects of antagonists on mean arterial pressure (MAP) and heart rate (HR) responses to several agonists in conscious rats

		Change in MAP (mmHg)			*Change in HR* (beats min^{-1})		
Blocker	*Agonist*	a	b	c	a	b	c
Phent	Mtx1	40 ± 6	0	0	−106 ± 14	0	0
Reserp	Tyram	46 ± 3[d]	17 ± 5	17 ± 5	−108 ± 11*	−16 ± 10	−21 ± 9
Prop	Isop	−37 ± 3	1 ± 1	17 ± 8	116 ± 13	2 ± 2	27 ± 9
Mecam	Mtx2	55 ± 2	101 ± 5	43 ± 8	−169 ± 17	0	0
Atrop	ACh	−44 ± 2	0	0	47 ± 2	0	0
Capt	AI	43 ± 4	0	15 ± 1	−83 ± 13	0	−28 ± 7

The effects (mean ± s.e.mean) of isoprenaline (Isop, 1 μg kg^{-1}), tyramine (Tyram, 200 μg kg^{-1}), methoxamine (Mtx1 20 μg kg^{-1}; Mtx2, 30 μg kg^{-1}), acetylcholine (ACh, 1 μg kg^{-1}) and angiotensin I (AI, 1 μg kg^{-1}) on MAP and HR were obtained before (a), 20 min after (b) the administrations of: phentolamine (Phent), propranolol (Prop), reserpine (Reserp), mecamylamine (Mecam), atropine (Atrop) or captopril (Capt) and, 2 h after (c) an i.v. bolus injection of N^G-nitro-L-arginine.
All results in (b) and (c) are significantly different from the corresponding control values (a) within the same group ($P < 0.05$). [d] The data were from the vehicle-treated rat group. $n = 6$ per group.

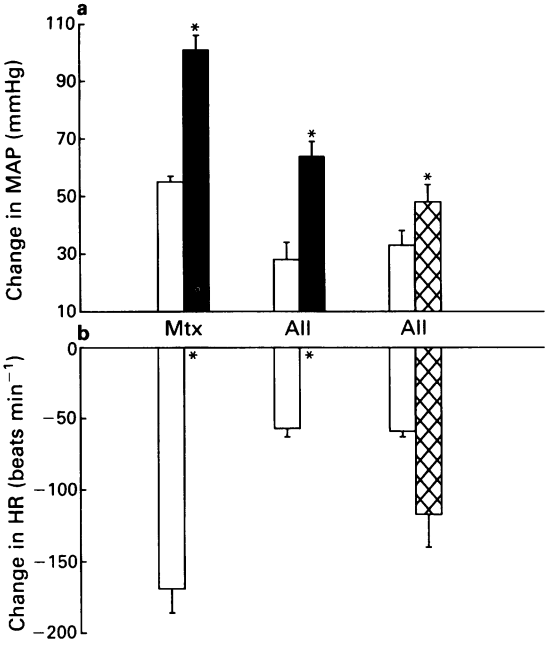

Figure 1 Effects of methoxamine (Mtx) and angiotensin II (AII) on mean arterial pressure (MAP) (a) and heart rate (HR) (b) before (open columns) and 20 min after the administrations of mecamylamine (filled columns) and phentolamine (cross hatched column). Values are means with s.e.mean shown by vertical bars; $n = 6$ in each group. * Represents significant difference from corresponding control values prior to the administration of an antagonist ($P < 0.05$).

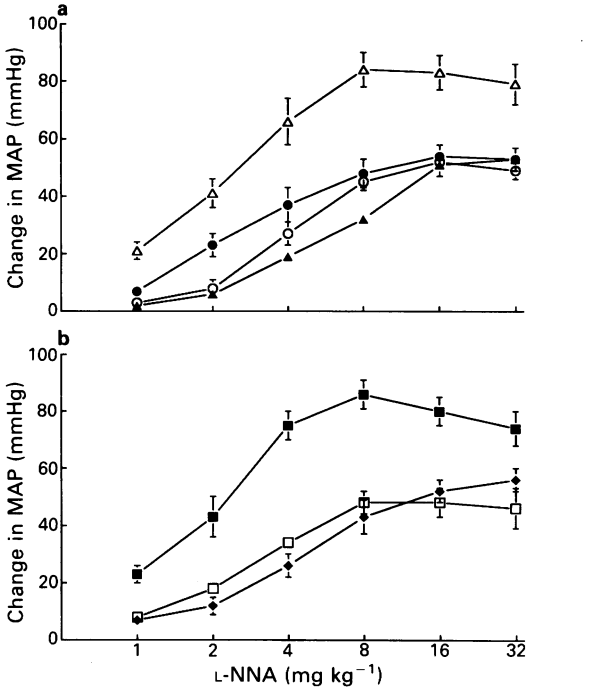

Figure 2 Dose-response curves (mean ± s.e.mean) of the effects of i.v. bolus doses of N^G-nitro-L-arginine (L-NNA) on mean arterial pressure (MAP) in groups ($n = 6$ each) of conscious rats pretreated with normal saline (○ in a), reserpine (● in a), phentolamine (△ in a), propranolol (▲ in a), atropine (□ in b), mecamylamine (■ in b) and captopril (◆ in b).

pressor effects and bradycardia induced by methoxamine throughout the study period. The pressor response to methoxamine was enhanced by mecamylamine (Group V) at 20 min but not altered at 2 h following the administration of L-NNA. The reflex bradycardia induced by methoxamine was abolished by mecamylamine throughout the experiments. Intravenous bolus doses of tyramine in vehicle-treated rats (Group I) increased MAP and decreased HR; in Group IV rats pretreated with reserpine tyramine caused markedly less pressor and bradycardic responses than in control rats. Isoprenaline (Group III) caused depressor and tachycardic responses; both were almost totally abolished at 20 min after the injection of propranolol and remained markedly attenuated 2 h after the administration of L-NNA. Atropine (Group VI) completely abolished the depressor and reflex tachycardic response of acetylcholine throughout the study period. At 20 min after the injection of captopril (Group VII), the pressure and bradycardic effects of angiotensin I were abolished. Both responses to angiotensin I remained attenuated 2 h after the injections of L-NNA.

Pressor responses to angiotensin II (Groups IX and X) and methoxamine (Group V) were potentiated by mecamylamine or phentolamine (Figure 1). Reflex bradycardia in response to angiotensin II and methoxamine was totally blocked by mecamylamine but reflex bradycardia to angiotensin II was unaffected by phentolamine.

Dose-response curves for N^G-nitro-L-arginine

Intravenous bolus doses of L-NNA in vehicle-treated rats dose-dependently increased MAP (Figure 2). Pretreatment with either mecamylamine or phentolamine potentiated the pressor response to L-NNA by reducing ED_{50} and increasing E_{max} values (Figure 2, Table 3). Pretreatment with the other antagonists used in this study did not significantly alter the dose-MAP response curves for L-NNA (Figure 2, Table 3).

Figure 3 shows the relationships between HR and MAP for rats in Groups I to VII. In vehicle-treated rats (Group I), MAP after L-NNA was negatively correlated with HR. Significant correlations of MAP with HR were also obtained in rats pretreated with phentolamine, propranolol, atropine or captopril but not with reserpine or mecamylamine (Table 4). The slope of the curve was not significantly altered by atropine, propranolol or captopril but was significantly increased by phentolamine. The intercept was decreased by propranolol,

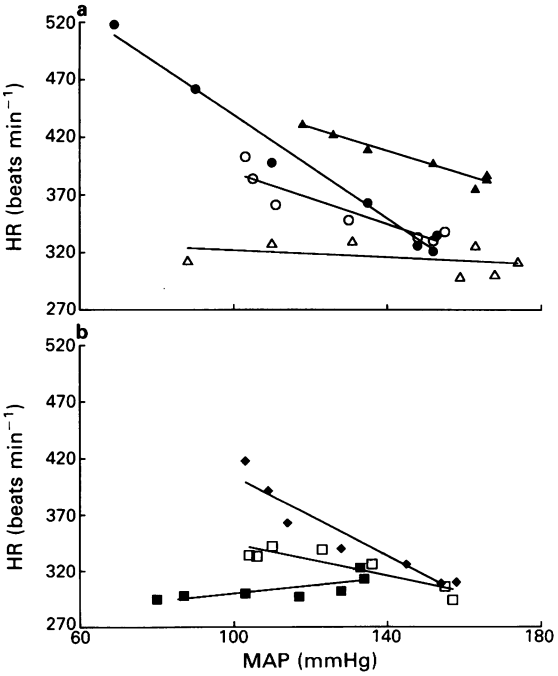

Figure 3 Relationship of heart rate (HR) (a) to mean arterial pressure (MAP) (b) after injection of N^G-nitro-L-arginine (L-NNA, 1–32 mg kg^{-1}, i.v. bolus) in conscious rats pretreated with normal saline (○ in a), phentolamine (● in a), mecamylamine (△ in a), atropine (▲ in a), propranolol (□ in b), reserpine (■ in b) and captopril (◆ in b). Each point represents mean values from six rats given the same dose of L-NNA.

Table 3 ED_{50} values and maximum effects (E_{max}) of N^G-nitro-arginine (L-NNA) on mean arterial pressure in conscious rats pretreated with normal saline, phentolamine, propranolol, reserpine, mecamylamine, atropine or captopril

Antagonist	ED_{50} (mg kg^{-1})	E_{max} (mmHg)
Normal saline	4.3 ± 0.8	52 ± 2
Phentolamine	2.1 ± 0.2*	87 ± 6*
Propranolol	6.3 ± 0.6	54 ± 3
Reserpine	3.1 ± 0.6	56 ± 4
Mecamylamine	1.9 ± 0.2*	86 ± 5*
Atropine	2.7 ± 0.3	51 ± 5
Captopril	5.0 ± 1.1	56 ± 4

All values represent mean ± s.e.mean. $n = 6$ per group. * Denotes significant difference from normal saline-treated group ($P < 0.05$).

Table 4 Slope, intercept and correlation coefficient (r) of the heart rate vs mean artery pressure curves of N^G-nitro-L-arginine (L-NNA, 1–32 mg kg^{-1}, i.v. bolus) in conscious rats ($n = 6$ per group) pretreated with normal saline, phentolamine, propranolol, reserpine, mecamylamine, atropine or captopril

Group	r	*Slope*	*Intercept*
Normal saline	0.85 ± 0.03[a]	−1.17 ± 0.24	509 ± 32
Phentolamine	0.96 ± 0.01[a]	−2.29 ± 0.08*	669 ± 23*
Propranolol	0.80 ± 0.05[a]	−0.75 ± 0.20	419 ± 20*
Reserpine	0.72 ± 0.06	[b]	[b]
Mecamylamine	0.46 ± 0.12	[b]	[b]
Atropine	0.83 ± 0.04[a]	−1.16 ± 0.22	557 ± 51
Captopril	0.89 ± 0.02[a]	−1.68 ± 0.14	570 ± 17

Slope (beats min^{-1} mmHg^{-1}), intercept (beats min^{-1}) and r values represent mean ± s.e.mean. [a] Denotes significance of r ($P < 0.05$); [b] values were not obtained due to insignificant correlation coefficient; * denotes significant difference from respective values in normal saline group ($P < 0.05$).

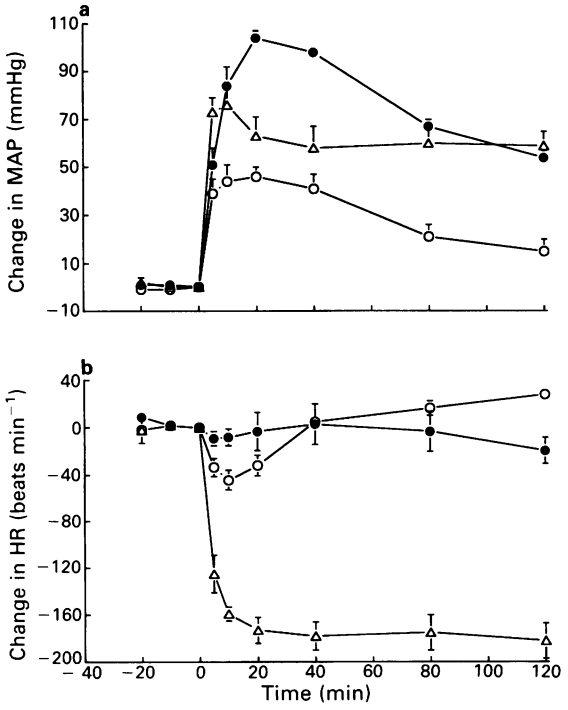

Figure 4 Time course of the effects of N^G-nitro-L-arginine (L-NNA, 32 mg kg^{-1}) on mean arterial pressure (MAP) (a) and heart rate (HR) (b) in groups ($n = 6$ each) of conscious rats pretreated with normal saline (○), mecamylamine (●) and phentolamine (△). Values are means with s.e.mean shown by vertical bars.

increased by phentolamine but not significantly altered by captopril and atropine.

Time course of the effects of N^G-nitro-L-arginine

In control rats given normal saline the MAP response to a single dose of L-NNA started almost immediately and reached a plateau 10 min after injection (Figure 4a). The rise phase $t_{1/2}$ was 4.8 min (geometric mean, 95% confidence limit: 2.0–11.6); MAP at 40 min was not different from MAP at 10 min and remained elevated 2 h after injection. Mecamylamine and phentolamine potentiated the peak MAP response to L-NNA (Figure 4a). Mecamylamine did not alter the rise phase $t_{1/2}$ (5.5 min, 95% confidence limit: 3.2–9.4) but phentolamine reduced it (to 1.5 min, 95% confidence limit: 1.0–2.3).

The pressor response to L-NNA was accompanied by initial significant decreases of HR at 5, 10, 20 min after injection followed by a recovery of HR and continual significant increases of HR at 80 and 120 min even when MAP was still above the control level (Figure 4b). Mecamylamine abolished the biphasic effects of L-NNA on HR. Phentolamine, on the other hand, potentiated and prolonged the bradycardia.

Discussion

Our results show that L-NNA is a potent and long-lasting pressor agent in conscious rats. Captopril and blockers of the autonomic nervous system, namely, mecamylamine, phentolamine, reserpine, propranolol and atropine, did not attenuate the pressor responses to L-NNA. This indicates that the pressor effect of L-NNA does not rely on the integrity of these two vasopressor systems. It has been reported that the pressor effects of L-MMA (Rees *et al.*, 1989b; Aisaka *et al.*, 1989) and L-NNA (Wang & Pang, 1990) are antagonized by L-Arg suggesting that N^G-substituted L-Arg analogues raise MAP via inhibiting NO synthase.

Pretreatment with mecamylamine potentiated MAP responses to L-NNA, angiotensin II and methoxamine. Phentolamine increased pressor responses to L-NNA and angiotensin II. Phentolamine but not mecamylamine, however, reduced the rise time $t_{1/2}$. This non-specific enhancement of the pressor effects of vasopressor agents after ganglionic or α-adrenoceptor blockade is consistent with well-known observations that acute pressor responses in intact animals are buffered by the simultaneous withdrawal of sympathetic tone to vascular smooth muscles (Lum & Rashleigh, 1961; Mawji & Lockett, 1963; Minson *et al.*, 1989).

MAP response to the injection of a single dose of L-NNA was associated with significant initial bradycardia (0 to 40 min) followed by tachycardia. A biphasic HR response to L-NNA was also observed in pentobarbitone-anaesthetized rats (Wang & Pang, 1990). Biphasic HR responses to L-MMA in chloralose and urethane anaesthetized rats have also been described (Togashi *et al.*, 1990). Mecamylamine abolished HR responses to L-NNA, suggesting that the biphasic HR response is mediated via reflex changes in the activities of the autonomic nervous system.

The tachycardic component was not seen in rats given cumulative doses of L-NNA, presumably due to the shorter observation time given to each dose of L-NNA. The results from cumulative dose-response relationships to L-NNA in the absence of an antagonist, show that the MAP effects of L-NNA are negatively correlated with HR. Treatment with mecamylamine or reserpine abolished the reflex changes in HR following alterations in MAP. The slope of the HR-MAP curve was slightly reduced by propranolol but unaffected by atropine. The lack of a correlation of HR to MAP after treatment with reserpine suggests that in conscious rats, inhibition of sympathetic nerve activity rather than potentiation of parasympathetic nerve activity is involved in reflex changes in HR. These results are consistent with the observation that bradycardia induced by L-MMA was associated with reduced renal

sympathetic nerve activity (Togashi *et al.*, 1990). Our results also show that phentolamine increased the slope of the curve. Phentolamine has been shown to increase markedly plasma levels of adrenaline and noradrenaline (Tabrizchi *et al.*, 1988). Therefore, enhanced reflex bradycardia in response to L-NNA in the presence of phentolamine may have been a consequence of elevated background sympathetic nerve activities as baseline HR was elevated by phentolamine.

In conclusion, the pressor effect of L-NNA does not rely on the integrity of the autonomic nervous system or the renin-angiotensin system. The biphasic changes of HR induced by L-NNA are attributable to reflex alterations in the activities of the autonomic nervous system.

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The effects of phosphoramidon on the regional haemodynamic responses to human proendothelin [1-38] in conscious rats

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1 Cardiovascular responses to human proendothelin [1–38], in the absence and presence of phosphoramidon, were studied in conscious Long Evans rats, chronically instrumented for the continuous recording of heart rate, systemic arterial blood pressure and renal, mesenteric and hindquarters blood flows.

2 A dose of 0.1 nmol kg^{-1} human proendothelin [1-38] caused a slight pressor effect (maximum 5 ± 2 mmHg), but a clear bradycardia (maximum -29 ± 7 beats min^{-1}). Renal haemodynamics were unchanged but there was mesenteric vasoconstriction and a vasodilatation followed by a vasoconstriction in the hindquarters.

3 A dose of 1.0 nmol kg^{-1} human proendothelin [1-38] caused a gradual hypertension (maximum 42 ± 4 mmHg at 10 min) and a profound bradycardia (-149 ± 10 beats min^{-1} at 30 min). There were gradual but marked, renal and hindquarters vasoconstrictions, whereas there was a substantial mesenteric vasoconstriction that was relatively rapid in onset.

4 In 2 animals, administration of human proendothelin [1–38] at a dose of 10 nmol kg^{-1} caused an initial hypotension followed by a rapidly-developing pressor effect; there were renal and mesenteric vasoconstrictions and vasodilatation followed by vasoconstriction in the hindquarters. These changes were very similar to those seen following injection of endothelin-1 (0.1 nmol kg^{-1}).

5 Phosphoramidon (2 μmol kg^{-1}) had no cardiovascular effects itself and it did not affect significantly the pressor or mesenteric vasoconstrictor effects of human proendothelin [1-38], but it reduced the bradycardia and renal and hindquarters vasoconstrictor responses. A higher dose of phosphoramidon (10 μmol kg^{-1}) caused significant attenuation of all the responses to human proendothelin [1-38], but a substantial mesenteric vasoconstrictor response still occurred under these conditions.

6 The results are consistent with the involvement of phosphoramidon-sensitive enzyme systems in the conversion of human proendothelin [1-38] to endothelin-1 *in vivo*. In addition, considering the different patterns of responses to human proendothelin [1-38] in the effector tissues studied (heart, and renal, mesenteric and hindquarters vascular beds), and the differential degrees of inhibition of them by phosphoramidon, it is likely that the effects of human proendothelin [1-38] were due to its local (rather than systemic) conversion to endothelin-1 by processes with differing degrees of susceptibility to phosphoramidon.

Keywords: Phosphoramidon; human proendothelin [1-38]; regional haemodynamics

Introduction

In their original paper, Yanagisawa *et al.* (1988) proposed that porcine proendothelin [1-39] was converted into endothelin (i.e. endothelin-1) by an endothelin-converting enzyme. Considering the unusual proteolytic processing involved, Yanagisawa *et al.* (1988) suggested the enzyme was likely to be an endopeptidase with a chymotrypsin-like specificity. Recently, both Matsumura *et al.* (1990) and Fukuroda *et al.* (1990) found that phosphoramidon caused substantial inhibition of the pressor effects of porcine proendothelin [1-39] and human proendothelin [1-38], respectively, in anaesthetized rats, indicating that a neutral endopeptidase-like, endothelin-converting enzyme system might be involved in the production of endothelin-1 *in vivo*. A similar conclusion was reached by McMahon *et al.* (1991).

We considered it feasible that the *in vivo* conversion of human proendothelin [1-38] to endothelin-1 might vary in different regional vascular beds and, in addition, that such processes might be differently sensitive to phosphoramidon. Therefore, in the present work we compared the regional haemodynamic effects of human proendothelin [1-38] in the same conscious rats in the absence and presence of phosphoramidon.

Methods

Male, Long Evans rats (350–450 g) were used in all experiments since our earlier studies on endothelin-1 (Gardiner *et al.*, 1990a,b) had used this strain. Under sodium methohexitone anaesthesia (60 mg kg^{-1} i.p. supplemented as required) miniaturized, pulsed Doppler probes (Haywood *et al.*, 1981) were implanted around the left renal and superior mesenteric arteries and the distal abdominal aorta, below the level of the ileocaecal artery. Following surgery, animals were given ampicillin (7 mg kg^{-1} i.m., Penbritin, Beechams) and returned to their home cages with free access to food and water for 7–14 days. After this time intravascular catheters (right jugular vein and distal abdominal aorta) were implanted under brief anaesthesia (sodium methohexitone, 40 mg kg^{-1} i.p.), and animals were then left to recover for at least 24 h before experiments were begun (Gardiner *et al.*, 1990c). The protocols were completed over 3 days. In preliminary experiments, human proendothelin [1-38] was given in i.v. bolus doses of 0.1, 1.0 and 10 nmol kg^{-1}. However, the pressor effects and bradycardia produced by the latter dose were very marked and the 2 animals which received it showed progressive cardiovascular deterioration. Therefore, only the 0.1 and 1.0 nmol kg^{-1} doses of human proendothelin [1-38] were used in the full experiment.

On the first experimental day i.v. bolus doses (0.1 and 1.0 nmol kg^{-1}) of human proendothelin [1-38] and of endothelin-1 (0.01 and 0.1 nmol kg^{-1}) were given at least 2 h apart, with the lower doses of both peptides being given first. Since the lower dose of human proendothelin [1-38] had only slight effects (see Results), on the second and third experimental days the higher dose (1 nmol kg^{-1} i.v. bolus) only of the peptide was given 5 min after injection of phosphoramidon in an i.v. bolus dose of either 2 or 10 μmol kg^{-1}. Animals

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received the 2 doses of phosphoramidon in random order on days 2 and 3.

Throughout an experiment, continuous recordings were made of phasic and mean systemic arterial blood pressures, instantaneous heart rate, and phasic and mean Doppler shift signals. Percentage changes in the latter were taken as indices of flow changes (Haywood *et al.*, 1981) and from the mean arterial pressure and mean Doppler shift signals, % changes in regional vascular conductances were calculated (Gardiner *et al.*, 1990a, b, c). The mean values quoted were derived from averaging (by eye) over 20 s around the times given, except in the case of values at zero time, in which case they refer to the 20 s period immediately prior to the intervention.

Data analysis

Changes relative to baseline were assessed by Friedman's test (Theodorsson-Norheim, 1987), while comparisons of responses to human proendothelin [1-38] in the absence and presence of phosphoramidon were compared by the Kruskal-Wallis test applied to areas under or over curves (AUC or AOC) obtained by computer analysis (Gardiner *et al.*, 1990c). A P value <0.05 was taken as significant. In the results AUC or AOC are given as means ± s.e.mean in arbitrary units.

Since we have previously described the haemodynamic effects of endothelin-1 (Gardiner *et al.*, 1989a,c; 1990a,b), full data are not repeated in this paper. However, we considered the comparison of the profiles of the responses to the 2 peptides in the same animals to be germane to interpretation of the results obtained with human proendothelin [1-38], and hence have referred to our current findings with endothelin-1 in the Discussion.

Drugs and peptides

Phosphoramidon (Sigma UK) was dissolved in saline (157 mmol l^{-1} NaCl) and human proendothelin [1-38] and endothelin-1 (Bachem UK) were dissolved in distilled water and then diluted in saline containing 1% bovine serum albumin (Sigma). Injections were given in 100 μl and flushed in with 100 μl saline. Concentrated aliquots of peptide solutions were stored at −80°C before being thawed and diluted for use.

Results

Absolute values for average resting cardiovascular variables under control conditions and after phosphoramidon are given in Table 1.

Responses to human proendothelin [1-38]

Bolus injections of vehicle (100 μl) had no consistent cardiovascular actions, except for slight tachycardias.

Human proendothelin [1-38] at a dose of 0.1 nmol kg^{-1} caused a small, but significant, pressor effect (at 5, 15 and 20 min; AUC 224 ± 49 units) accompanied by a marked bradycardia (AOC, 1403 ± 187 units) (Table 2). Renal blood flow showed no significant change, mesenteric blood flow a modest, but significant, reduction (AOC, 461 ± 87 units), while in the hindquarters there was an initial increase (AUC, 174 ± 94 units) followed by a delayed decrease (AOC, 483 ± 107 units) in flow (Table 2). Changes in calculated vascular conductances indicated a renal vasoconstriction (AOC, 346 ± 80 units), a more marked, earlier-onset, mesenteric vasoconstriction (AOC, 626 ± 79 units) and a transient vasodilatation (AUC, 156 ± 103 units) followed by a vaso-

Table 1 Cardiovascular variables (on 3 different experimental days) in the same conscious, Long Evans rats under control conditions or 5 min after injection of phosphoramidon

		Control	*Phosphoramidon* (2 μmol kg^{-1})	*Phosphoramidon* (10 μmol kg^{-1})
	Heart rate (beats min^{-1})	333 ± 8	335 ± 6	333 ± 10
	Mean arterial blood pressure (mmHg)	105 ± 4	107 ± 2	107 ± 4
Flow (kHz)	Renal	10.2 ± 1.0	9.8 ± 1.6	10.0 ± 1.2
	Mesenteric	7.0 ± 0.6	7.7 ± 0.6	7.1 ± 0.6
	Hindquarters	4.7 ± 0.7	4.3 ± 0.8	4.3 ± 0.7
Conductance ([kHz $mmHg^{-1}$]10^3)	Renal	96 ± 7	92 ± 14	93 ± 10
	Mesenteric	68 ± 7	73 ± 7	68 ± 8
	Hindquarters	46 ± 8	41 ± 8	41 ± 7

Values are mean ± s.e.mean. $n = 8$.

Table 2 Cardiovascular changes following an i.v. bolus dose (0.1 nmol kg^{-1}) of human proendothelin [1-38] in conscious, Long Evans rats

	Time (min)	1	5	10	15	20	30	60
	Heart rate (beats min^{-1})	13 ± 6	−8 ± 4	−20 ± 4*	−22 ± 2*	−25 ± 3*	−29 ± 7*	−13 ± 7
	Mean arterial blood pressure (mmHg)	2 ± 1	5 ± 2*	4 ± 2	5 ± 2*	4 ± 1*	3 ± 1	2 ± 1
Flow (%)	Renal	1 ± 1	1 ± 2	−1 ± 4	−3 ± 3	−4 ± 3	−1 ± 3	0 ± 4
	Mesenteric	−7 ± 2	−4 ± 2	−5 ± 3	−9 ± 2*	−8 ± 2*	−9 ± 2*	0 ± 6
	Hindquarters	17 ± 5*	0 ± 3	−2 ± 2	−1 ± 4	−6 ± 4	−9 ± 2*	−4 ± 5
Conductance (%)	Renal	−1 ± 1	−4 ± 2	−5 ± 3	−8 ± 3*	−8 ± 2*	−5 ± 2*	−3 ± 4
	Mesenteric	−8 ± 3	−9 ± 3*	−9 ± 2*	−13 ± 2*	−12 ± 2*	−11 ± 2*	−2 ± 6
	Hindquarters	15 ± 5*	−5 ± 4	−6 ± 3	−5 ± 5	−10 ± 3*	−12 ± 2*	−6 ± 6

Values are mean ± s.e.mean. $n = 8$.
* $P < 0.05$ versus baseline (Friedman's test)

constriction (AOC, 555 ± 117 units) in the hindquarters (Table 2).

Following administration of human proendothelin [1-38] at a dose of 1 nmol kg^{-1} there was a marked pressor response which reached a maximum after about 10 min (AUC, 1990 ± 160 units) (Figures 1 and 2). Initially there was a slight tachycardia (AUC, 22 ± 9 units) (similar to that seen after injection of vehicle) but thereafter, a substantial and sustained bradycardia occurred (AOC, 7187 ± 551 units) (Figures 1 and 2). Renal blood flow showed a gradual, but marked, reduction (AOC, 2072 ± 146 units), while there was a fall in mesenteric blood flow (AOC, 2806 ± 127 units) that was more rapid in onset and significantly greater ($P < 0.05$) at its nadir (Figure 2). There was a slight and non-significant rise in hindquarters blood flow initially (AUC, 42 ± 9 units), but subsequently there was a progressive and sustained fall (AOC, 3056 ± 161 units). The changes in regional vascular conductances followed similar patterns to the flows (renal AOC, 2960 ± 106 units; mesenteric AOC, 3509 ± 125 units; hindquarters AUC, 29 ± 7 units, AOC, 3730 ± 136 units) (Figure 3).

Human proendothelin [1-38] at a dose of 10 nmol kg^{-1} was administered to 2 animals only because it caused marked cardiovascular deterioration over the ensuing 1 h. However, in the animals that received this dose of the peptide there was an initial depressor response and a tachycardia. Subsequently, mean arterial blood pressure increased and bradycardia developed (Figure 4). There were rapid reductions in renal and mesenteric blood flows (Figure 4). Initially there was an increase in hindquarters blood flow, but thereafter a substantial reduction occurred (Figure 4).

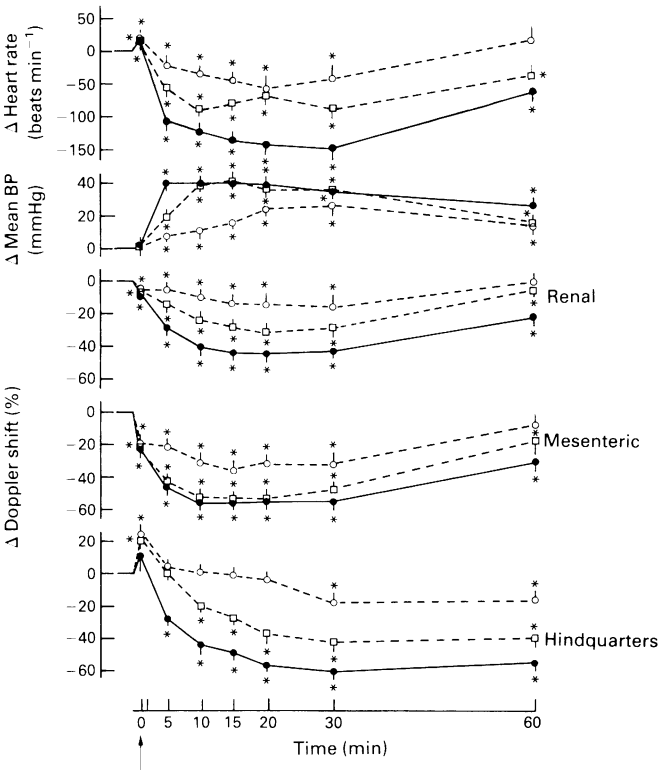

Figure 2 Group data for the cardiovascular responses to human proendothelin [1-38] at a dose of 1 nmol kg^{-1} (i.v. bolus given at the arrow) on 3 different experimental days: (●) shows the responses to human proendothelin [1-38] alone; (□) the responses 5 min after a 2 μmol kg^{-1} bolus dose of phosphoramidon; (○) the responses 5 min after a 10 μmol kg^{-1} bolus dose of phosphoramidon. Values are mean and bars show s.e.mean ($n = 8$). * $P < 0.05$ versus baseline (Friedman's test).

Responses to human proendothelin [1-38] in the presence of phosphoramidon

Phosphoramidon (2 and 10 μmol kg^{-1}) had no significant effects on resting cardiovascular status (Table 1).

In the presence of phosphoramidon at a dose of 2 μmol kg^{-1}, the overall pressor effect of a dose of 1 nmol kg^{-1} of human proendothelin [1-38] was unchanged (AUC, 1473 ± 222 units), but the bradycardia was reduced (AOC, 3651 ± 731 units) (Figure 2). There were significant ($P < 0.05$) attenuations of the falls in renal and hindquarters, but not in mesenteric blood flows (renal AOC, 1143 ± 193 units; hindquarters AOC, 1751 ± 269 units; mesenteric AOC, 2226 ± 335 units) and vascular conductances (renal AOC, 1991 ± 291 units; hindquarters AOC, 2381 ± 345 units; mesenteric AOC, 2724 ± 401 units) (Figures 2 and 3). In addition, there were significant, initial increases in hindquarters flow

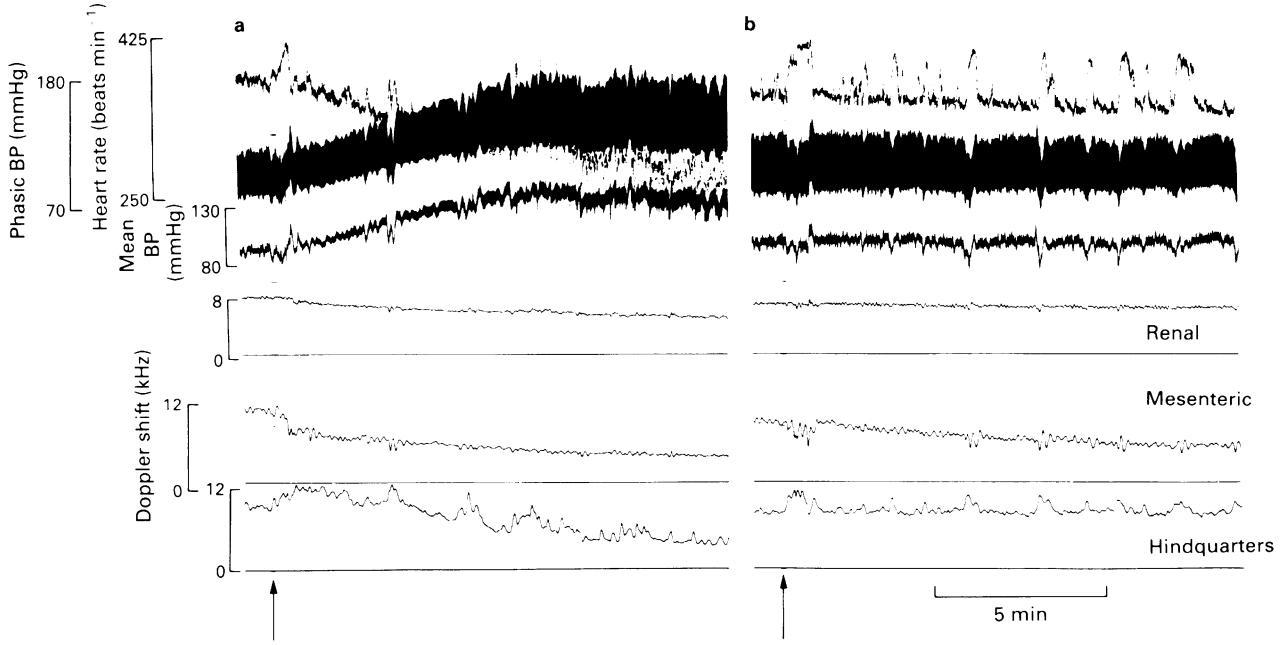

Figure 1 Cardiovascular responses to human proendothelin [1-38] at a dose of 1.0 nmol kg^{-1} (given by i.v. bolus at the arrows) in the same conscious, Long Evans rat on 2 different experimental days: (a) shows the responses to human proendothelin [1-38] alone, while (b) shows the responses 5 min after phosphoramidon (10 μmol kg^{-1} i.v. bolus). In this animal the effect of phosphoramidon on the responses to proendothelin [1-38] were particularly marked.

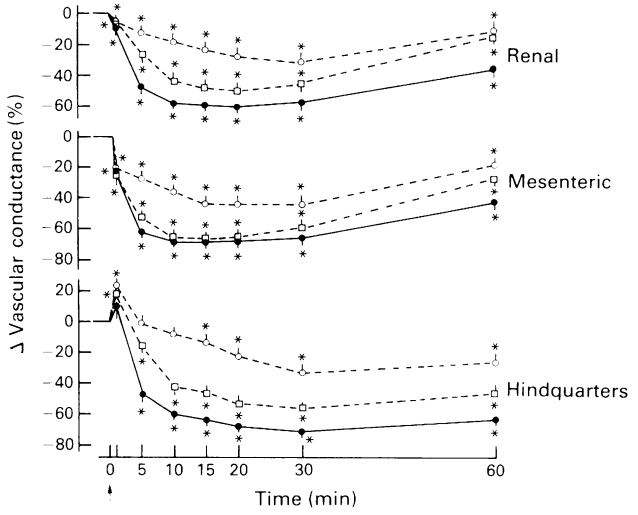

Figure 3 Group data (from the results in Figure 2) for the calculated vascular conductance responses to human proendothelin [1-38] at a dose of 1 nmol kg^{-1} (i.v. bolus given at the arrow) on 3 different days: (●) shows the responses to human proendothelin [1-38] alone; (□) the responses 5 min after a 2 μmol kg^{-1} bolus dose of phosphoramidon; (○) the responses 5 min after a 10 μmol kg^{-1} bolus dose of phosphoramidon. Values are mean and bars show s.e.mean (n = 8). * $P < 0.05$ versus baseline (Friedman's test).

(AUC, 70 ± 17 units) and vascular conductance (AUC, 49 ± 17 units) (Figures 2 and 3), that were significantly ($P < 0.05$) greater than those in the absence of phosphoramidon.

Following injection of phosphoramidon at a dose of 10 μmol kg^{-1}, there was significant ($P < 0.05$) attenuation of the pressor effect of human proendothelin [1-38] (AUC, 1104 ± 123 units) and the bradycardia was further reduced (AOC, 1971 ± 362 units) (Figures 1 and 2), as were the falls in renal and hindquarters flows (renal AOC, 616 ± 134 units; hindquarters AOC 752 ± 168 units) and conductances (renal AOC 1359 ± 148 units; hindquarters AOC 1372 ± 174 units) (Figures 1–3). Indeed, in the latter vascular bed the reduction in flow was not significant until 30 min after injection of human proendothelin [1-38], and hence was significantly delayed relative to the control response, as was the change in vascular conductance, although this occurred slightly earlier (Figures 2 and 3). As in the presence of the lower dose of phosphoramidon, there were initial increases in hindquarters flow (AUC, 190 ± 82 units) and vascular conductance (AUC, 86 ± 27 units) following administration of proendothelin [1-38] that were significantly ($P < 0.05$) greater than the changes in the absence of phosphoramidon.

Although there were significant ($P < 0.05$) attenuations of the overall falls in mesenteric flow (AOC, 1557 ± 175 units) and vascular conducance (2192 ± 109 units) in response to human proendothelin [1-38] in the presence of phosphoramidon at 10 μmol kg^{-1}, it is notable that the initial sharp reductions (i.e. at the 1 min time point) in these variables following injection of the peptide were unchanged relative to the control condition (Figures 2 and 3), and, furthermore, the residual responses were significantly ($P < 0.05$) greater than in the renal or hindquarters vascular beds (Figures 1–3).

Discussion

The present results corroborate recent findings showing that the neutral endopeptidase inhibitor, phosphoramidon, causes significant inhibition of the pressor effects of human proendothelin [1-38] (Fukuroda *et al.*, 1990a). In addition, our results indicate important regional differences in the haemodynamic effects of human proendothelin [1-38] in conscious rats and demonstrate clearly that these different regional effects are differentially sensitive to phosphoramidon, even under conditions in which the overall pressor effect of the peptide is unchanged.

Although phosphoramidon is a relatively selective inhibitor of neutral endopeptidase 24.11, this enzyme, or forms thereof, is capable of acting upon enkephalins, substance P, atrial natriuretic peptide, brain natriuretic peptide and bradykinin, at least (Ura *et al.*, 1987; Sybertz *et al.*, 1990; Vanneste *et al.*,

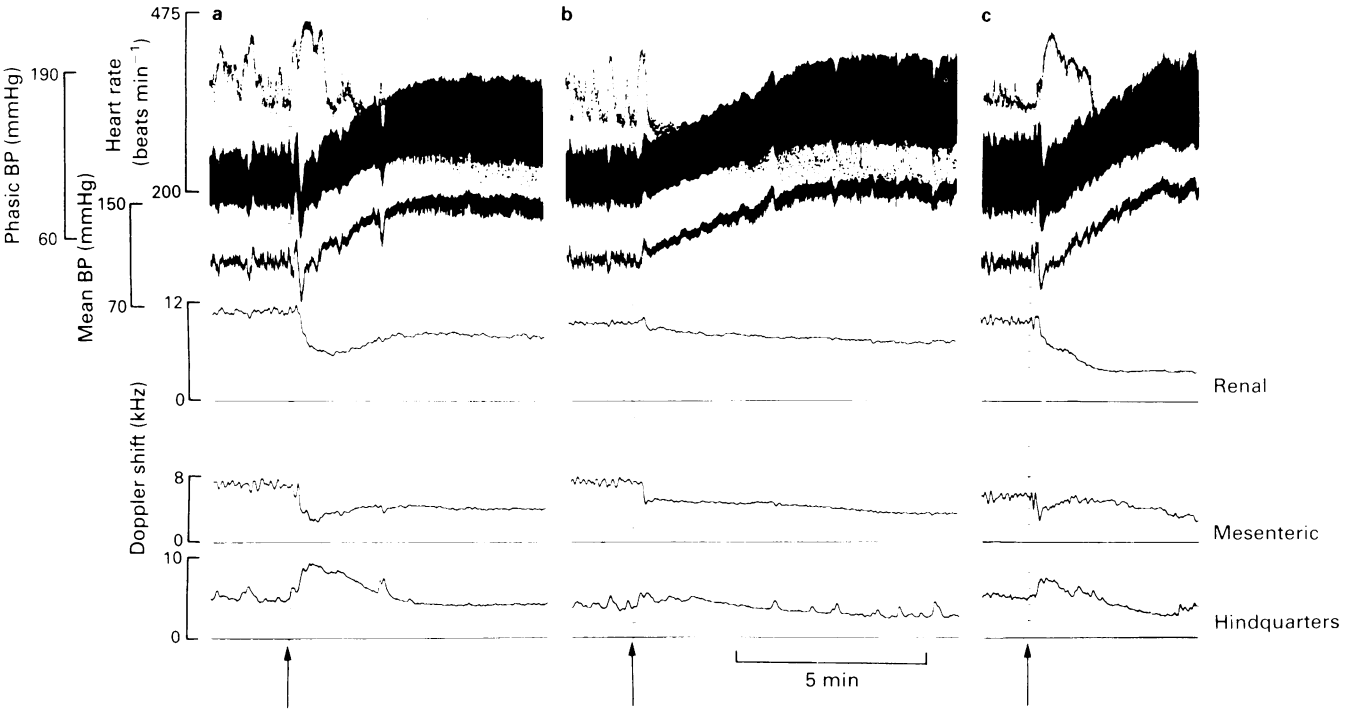

Figure 4 Cardiovascular changes in conscious Long Evans rats: (a) shows the response to endothelin-1 (0.1 nmol kg^{-1}); (b) the response to human proendothelin [1-38] (1 nmol kg^{-1}) in the same animal as in (a) and (c) the response to human proendothelin [1-38] at a dose of 10 nmol kg^{-1}.

1990; Gerbes & Vollmar, 1990). Hence, one needs to consider the possibility that the *in vivo* effects of phosphoramidon on the responses to human proendothelin [1-38] did not result from the inhibition of the peptide conversion to endothelin-1, but were due to inhibition of degradation of other endogenous peptides. However, this seems unlikely for several reasons. Firstly, it has been shown that the cardiovascular responses to inhibition of neutral endopeptidase are unaffected by a bradykinin antagonist (Sybertz *et al.*, 1990). Secondly, in the experiments of Sybertz *et al.* (1990) it was observed that inhibition of neutral endopeptidase in rats with DOCA-salt hypertension was associated with a rise in plasma levels of atrial natriuretic peptide and a fall in blood pressure. However, in the present experiments administration of phosphoramidon alone had no significant effect on cardiovascular variables. Thirdly, in the conscious, normotensive rat model used in the present experiments bolus injections of exogenous atrial natriuretic peptide cause tachycardia, hypotension, renal and mesenteric vasoconstrictions and hindquarters vasodilatation (Gardiner *et al.*, 1988), and infusions of atrial natriuretic peptide evoke renal, mesenteric and hindquarters vasoconstrictions (Gardiner *et al.*, 1989b). Thus, it seems unlikely that phosphoramidon-induced accumulation of endogenous atrial natriuretic peptide in our animals could explain the attenuation of the regional vasoconstrictor responses to human proendothelin [1-38]. Nonetheless, *in vivo*, factors other than inhibition of endothelin-converting enzyme(s) should not be dismissed. In this respect it is of significance that Sokolovsky *et al.* (1990) showed that endothelin-1 itself is degraded *in vitro* by a phosphoramidon-sensitive neutral endopeptidase. Thus *in vivo*, phosphoramidon might inhibit both the conversion of human proendothelin [1-38] to endothelin-1 and the degradation of the latter. Furthermore, it is feasible that any given dose of phosphoramidon could influence these processes to different extents.

The results obtained are discussed with reference to the variables measured.

Effects on heart rate

In conscious, intact rats, human proendothelin [1-38] at a dose of 0.1 nmol kg^{-1} caused significant bradycardia even when mean arterial blood pressure was unchanged (Table 2). Moreover, the bradycardia induced by the higher dose of the peptide was attenuated by phosphoramidon at a dose (2 μmol kg^{-1}) that had no influence on the pressor effects of human proendothelin [1-38]. These results indicate that the generation of endothelin-1 from the latter peptide might exert a selective negative chronotropic effect, but the possibility that this is due to cardiac conversion of human proendothelin [1-38] to endothelin-1 and a local effect of the latter on sinoatrial rate and/or on cardiac reflex mechanisms would need to be examined in other experiments.

Effects on mean arterial blood pressure

The gradual onset of the pressor action of human proendothelin [1-38] is consistent with conversion to endothelin-1 being necessary for the effect, as is its inhibition by phosphoramidon. To compare, formally, the rate of onset of this pressor response to that of endothelin-1 is difficult because of the pronounced initial depressor response to the latter (Yanagisawa *et al.*, 1988). Although administration of both peptides to the same animals can give the impression that the pressor action of endothelin-1 is more rapid in onset (Figure 4), this is probably a dose-related phenomenon, since, in the 2 animals that were given human proendothelin [1-38] at a dose of 10 nmol kg^{-1}, the pressor effect developed rapidly (Figure 4). Hence, the rate of conversion of exogenous human proendothelin [1-38] to endothelin-1 may be determined by the accessibility of the former peptide to the converting enzyme(s).

Several groups have claimed that human proendothelin [1-38] does not cause an initial depressor effect (Kashiwabara *et al.*, 1989; D'Orléans Juste *et al.*, 1990; Fukuroda *et al.*, 1990), but in recent experiments by Douglas & Hiley (1991), it was shown that human proendothelin [1-38] can cause an initial hypotension, the threshold for the response being about 100 fold higher than that for endothelin-1. In absolute terms, Douglas & Hiley (1991) found a dose of 1 nmol kg^{-1} of human proendothelin [1-38] to cause a mean fall of about 7 mmHg. In the present work the highest dose of this peptide used in the full experiments was 1 nmol kg^{-1}, and whilst there was no fall in blood pressure in the group mean data, nonetheless, in some animals there was a slight initial hypotensive response, and in the 2 animals that received the 10 nmol kg^{-1} dose of human proendothelin [1-38], there was an early depressor effect of about 16 mmHg (Figure 4). From these results it is not possible to deduce which peptide moiety was responsible for triggering this event, but the finding that it could occur in some animals when human proendothelin [1-38] was administered in the presence of phosphoramidon (Figure 1) might indicate an involvement of human proendothelin [1-38] itself in this depressor response.

Effects on renal haemodynamics

The gradual-onset reductions in renal blood flow following administration of 1.0 nmol kg^{-1} human proendothelin [1-38] were dose-dependently attenuated by phosphoramidon. This finding, together with the lack of effect of a dose of 0.1 nmol kg^{-1} proendothelin [1-38] on renal blood flow, when flows in the other vascular beds changed, is consistent with the renal vascular effects of human proendothelin [1-38] being due to local, rather than systemic, conversion to endothelin-1. These results contrast with those of Hoffman *et al.* (1990) who found that porcine proendothelin [1-38] did not reduce renal blood flow or vascular conductance in conscious Sprague-Dawley rats.

In the present work the slowly developing, renal vasoconstrictor effect of human proendothelin [1-38] at a dose of 1.0 nmol kg^{-1} could have been due to a slow conversion to endothelin-1 and/or a relative insensitivity of this vascular bed to any endothelin-1 locally generated from the precursor peptide, since low doses of exogenous endothelin-1 cause only slight renal vasoconstriction at a time when mesenteric vasoconstriction is substantial (Gardiner *et al.*, 1989a; 1990a,b), and this difference persists in the presence of indomethacin (Gardiner *et al.*, 1990b). However, with higher doses of exogenous endothelin-1 the renal vascular bed shows a prompt and marked vasoconstriction, similar to that seen in the mesenteric vascular bed (Gardiner *et al.*, 1989a; 1990a,b; Figure 4). Furthermore, the 2 animals that received human proendothelin [1-38] at a dose of 10 nmol kg^{-1} in the present study showed a renal vasoconstriction that developed rapidly (Figure 4). Thus, it seems more likely that a relative insensitivity of the renal vascular bed to low levels of endothelin-1, rather than slow conversion of human proendothelin [1-38] to endothelin-1, explains the profile of renal vascular response to human proendothelin [1-38] at a dose of 1.0 nmol kg^{-1}.

Since the dynamics of the conversion of human proendothelin [1-38] to human endothelin-1 seem to be dependent on substrate availability, it appears that any regional differences in the responses to human proendothelin [1-38] cannot be taken as solid evidence for regional differences in converting enzyme activity.

Effects on mesenteric haemodynamics

The response of the mesenteric vascular bed to human proendothelin [1-38] was notable for several reasons: (1) this vascular bed showed clear reductions in flow and conductance in response to a dose of the peptide (0.1 nmol kg^{-1}) that had no significant renal haemodynamic, and only minimal pressor, effects: (2) this vascular bed showed rapid-onset reductions in blood flow, rather than the gradual changes seen in the renal and hindquarters vascular beds in response to the peptide (at a dose of 1.0 nmol kg^{-1}); (3) the early (i.e. measured at 1 min)

responses to the peptide were unaffected by either dose of phosphoramidon, and even in the presence of the highest dose of phosphoramidon used there was still a substantial mesenteric vasoconstrictor effect of human proendothelin [1-38]. These findings are consistent with the greater responsiveness of the mesenteric vascular bed to low doses of exogenous endothelin-1 (Gardiner *et al.*, 1989a; 1990a,b). It is feasible that the initial, rapid response to human proendothelin [1-38] was due to this peptide interacting with receptors in the mesenteric vascular bed, or to particularly rapid conversion of the peptide into endothelin-1 at that site (see Douglas & Hiley, 1991). The latter might occur if the endothelin converting enzyme(s) was especially active in that vascular bed, and could also explain the relative resistance of the mesenteric haemodynamic effects of human proendothelin [1-38] to phosphoramidon. However, it is possible that conversion of human proendothelin [1-38] to endothelin-1 in the mesenteric vascular bed also involves phosphoramidon-insensitive mechanisms.

Effects on hindquarters haemodynamics

The lower dose of human proendothelin [1-38] caused an initial increase in hindquarters flow and vascular conductance followed by a gradual vasoconstriction, whereas the higher dose of the peptide caused an initial increase in flow and vascular conductance only in the presence of phosphoramidon, when the subsequent vasoconstrictor response was dose-dependently attenuated. These observations are consistent with the vasodilator and vasoconstrictor responses being due to separate mechanisms that oppose each other, as is the case with exogenous endothelin-1 (Gardiner *et al.*, 1989a; 1990a,b). While the vasoconstrictor effects of endothelin-1 are reasonably well-characterized, the vasodilator effects are not, but it seems that, *in vivo*, neither cyclooxygenase products nor nitric oxide are involved (Gardiner *et al.*, 1989c; 1990b; Ohlstein *et al.*, 1990). Recent studies by Ohlstein *et al.* (1990) indicate that the hindquarters vasodilator effects of endothelin-1 are not demonstrable in the absence of blood and hence may depend on a blood-derived factor. Whatever the explanation, it appears that human proendothelin [1-38] can elicit this effect, and more so in the presence of phosphoramidon. However, human proendothelin [1-38] caused relatively slight initial hindquarters vasodilatation but substantial vasoconstriction, accompanied by reductions in blood flow below baseline levels. This picture differs from that seen with exogenous endothelin-1 which causes substantial initial increases in hindquarters flow and vascular conductance, but little subsequent reduction (Gardiner *et al.*, 1989a,c; 1990a,b; Figure 4). These results indicate that the gradual, but substantial, hindquarters vasoconstrictor effects of human proendothelin [1-38] were probably due to its local conversion to endothelin-1, and that this process does not activate vasodilator mechanisms as effectively as do bolus injections of exogenous endothelin-1 (Figure 4). One possible explanation of this difference is that, following administration of human proendothelin [1-38], endothelin-1 is generated close to the vessel wall and hence does not stimulate release of blood-derived vasodilator products (Ohlstein *et al.*, 1990) as effectively as do bolus injections of endothelin-1.

The delayed hindquarters vasoconstriction seen in response to human proendothelin [1-38] in the presence of the higher dose of phosphoramidon could have been due to several factors, including: (1) phosphoramidon inhibiting the degradation of endothelin-1 (Sokolovsky *et al.*, 1990) more effectively than the generation of endothelin-1 from the larger peptide, (2) accumulation of the other peptides (see above), and (3) the effects of phosphoramidon waning. These questions need to be addressed in future experiments.

In conclusion, the present results indicate that the pressor action of proendothelin [1-38] in conscious rats can be inhibited by phosphoramidon. However, the degree of inhibition of the regional haemodynamic effects of proendothelin [1-38] differs in different vascular beds, consistent with its effects being due to local conversion to endothelin-1 by mechanisms that show varying susceptibility to inhibition by phosphoramidon.

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Potencies of antagonists indicate that 5-HT_{1C} receptors mediate 1-3(chlorophenyl)piperazine-induced hypophagia

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1 1-3(Chlorophenyl)piperazine (mCPP) (5 mg kg^{-1}, i.p.) inhibited 2 h food intake in rats previously deprived of food for one day. Ten 5-hydroxytryptamine (5-HT) antagonists given s.c. opposed this hypophagic response. Calculated ID_{50} values correlated significantly with reported affinities ($r = 0.81$, $n = 10$, $P < 0.01$) for 5-HT_{1C} but not for 5-HT_2, 5-HT_{1A}, 5-HT_{1B} or 5-HT_{1D} receptors.

2 ID_{50} values of the ten antagonists against 5-hydroxytryptophan (5-HTP) + carbidopa-induced head shakes (a 5-HT_2-mediated response) correlated significantly ($r = 0.81$, $n = 10$, $P < 0.01$) with their affinities for 5-HT_2 but not for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} or 5-HT_{1D} receptors.

3 ID_{50} values for inhibition of hypophagia and head shakes did not correlate significantly with each other.

4 Ratios of ID_{50} values against hypophagia and 5-HT_2-mediated head shakes gave indices of relative *in vivo* potencies independent of differences in drug metabolism and disposition. These ratios correlated highly significantly ($r = 0.91$, $n = 10$, $P < 0.001$) with the ratios of the affinities of the drugs for 5-HT_{1C} (but not for 5-HT_{1A}, 5-HT_{1B} or 5-HT_{1B} or 5-HT_{1D} receptors) and with their affinities for 5-HT_2 receptors. These results strongly support the hypothesis that mediation of mCPP-induced hypophagia is by stimulation of 5-HT_{1C} receptors and the mediation of 5-HTP-induced head twitches by 5-HT_2 receptors.

Keywords: 5-HT_{1C} receptors, 5-HT_2 receptors, hypophagia, 1-3(chlorophenyl)piperazine (mCPP)

Introduction

1-3(Chlorophenyl)piperazine (mCPP) and 1-[3-(trifluoromethyl)phenyl]piperazine (TFMPP) cause hypophagia in food-deprived (Samanin *et al.*, 1979; Kennett & Curzon, 1988a) and freely feeding (Kennett *et al.*, 1987) rats. The drugs also cause hypolocomotion (Lucki *et al.*, 1989; Kennett & Curzon, 1988b) and anxiety-like behaviour in rat models (Kennett *et al.*, 1988). These effects are unlikely to account for the hypophagia as infusion of TFMPP into the paraventricular nucleus of the hypothalamus (PVN), a site associated with control of feeding (Shor-Posner *et al.*, 1986), causes hypophagia without hypolocomotion (Hutson *et al.*, 1988). Also, chlordiazepoxide prevented the anxiogenic effect of mCPP but neither its hypophagic nor hypolocomotor actions (Kennett *et al.*, 1988).

Since TFMPP- and mCPP-induced hypophagias are pharmacologically similar (Kennett & Curzon, 1988a) the hypophagic response to mCPP is also likely to be mediated by the PVN. Indeed, we have previously proposed that the hypophagic responses to both drugs are the result of 5-HT_{1C} receptor activation (Kennett & Curzon, 1988a). These sites appear to be located postsynaptically as mCPP-induced hypophagia is not blocked by raphe lesions (Samanin *et al.*, 1979). However, a problem in the investigation of the roles of 5-HT_{1C} receptors is that most antagonists with high affinity for them also have high affinity for 5-HT_2 receptors (Hoyer, 1988), as do many agonists such as quipazine and 1-(2,5-dimethoxy-4-iodophenyl)-L-aminopropane (DOI) (Schoeffter & Hoyer, 1989). Furthermore, both receptors share the same secondary messenger system (phosphoinositide hydrolysis) (Conn & Sanders-Bush, 1987). These similarities presumably reflect the 78% sequence homology of the two receptors (Hartig, 1989).

A number of publications have suggested that activation of 5-HT_2 receptors causes hypophagia. Thus the effects of antagonists on hypophagias induced by the 5-hydroxytryptamine (5-HT) releasing agent fenfluramine (Hewson *et al.*, 1988), the 5-HT_{1C}/5-HT_2 agonists DOI (Schechter & Simansky, 1988) and quipazine (Hewson *et al.*, 1989) have all been interpreted in terms of activation of 5-HT_2 receptors. Also, other reports suggest that the effect of TFMPP is 5-HT_2-mediated (Klodzinska & Chojnacka-Wojcik, 1990) and that the action of mCPP might not be 5-HT_{1C}-dependent (Aulakh *et al.*, 1989). These apparent conflicts with our findings may derive from experimental differences and from the customary use of *in vitro* affinity constants of antagonists as indices of receptor blockade *in vivo*. However, affinities *in vitro* do not necessarily closely indicate *in vivo* potencies as these could be influenced by drug absorption, metabolism and disposition.

We have now minimized the above problems by comparing the ratios of *in vivo* ID_{50} values of ten antagonists against mCPP-induced hypophagia and 5-HT_2-mediated (Bedard & Pycock, 1977; Yap & Taylor, 1983; Niemegeers *et al.*, 1983) head shakes with the corresponding ratios of *in vitro* drug affinities for various 5-HT_1 receptor subtypes and 5-HT_2 receptors. This provides indices of blockade of different 5-HT receptors which are independent of differences in drug absorption or metabolism and which can be compared with published *in vitro* affinity ratios. Results were consistent with our proposal (Kennett & Curzon, 1988a) that mCPP induces hypophagia by activating 5-HT_{1C} receptors and not 5-HT_2 receptors.

A preliminary account of this work has been presented (Kennett *et al.*, 1990).

Methods

Animals

Male Sprague Dawley rats (250–300 g, Charles River, UK) were housed individually with free access to food [Special Diet Services Ltd., Essex, England, Rm (IE) rodent diet] and water at 21 ± 2°C under a 12 h light/dark cycle (lights on 06 h 00 min) for at least 5 days prior to experimentation.

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Head shake response

Rats were placed in individual perspex cages (26 cm × 26 cm × 26 cm) at least 1 h prior to experimentation and injected with 25 mg kg^{-1} i.p. carbidopa between 13 h 00 min and 13 h 30 min. Antagonists or vehicle were injected s.c. immediately afterwards. 5-HTP 100 mg kg^{-1}, i.p., was injected 30 min later and the number of head shakes counted over 2 min periods 30, 60, 90 and 120 min later. Scores were then summed.

1-3(Chlorophenyl)piperazine-induced hypophagia

Rats were deprived of food but not water commencing between 16 h 00 min and 18 h 00 min. On the following day between 12 h 00 min and 13 h 00 min they were injected with antagonists, s.c. Twenty min later they were injected with either mCPP 5 mg kg^{-1} or saline i.p. After a further 20 min a weighed amount of food was placed in the food hoppers and food intake measured after 1 and 2 h. The data obtained at 1 h were not presented as they were largely consistent with the 2 h findings but less clearly defined.

Drugs

The 5-HT antagonists altanserin, ketanserin tartarate (Janssen, Beerse, Belgium) methysergide hydromaleate, pizotifen hydrogen maleate (Sandoz, Basle, Switzerland) metergoline (Farmitalia) and mianserin hydrochloride (Organon, Newhouse, U.K.) were dissolved in 10% acetic acid and made up to volume with 0.9% NaCl before bringing to pH 6.5 with 5 N NaOH. 1 n-Naphthyl piperazine hydrochloride (1-NP), mCPP dihydrochloride (both Research Biochemicals Inc., Wayland, U.S.A.), 4-isopropyl-7-methyl-9-(2-hydroxy-1-methylpropoxycarbonyl) 4,6,6A,7,8,9,10,10A-octahydro-indolo(4,3-f,g)quinoline maleate (LY 53857, Cohen *et al.*, 1983) (Lilly, Minneapolis, U.S.A.) and (±)-propranolol hydrochloride (ICI, Macclesfield, U.K.) were dissolved in 0.9% NaCl while ritanserin (Janssen, Beerse, Belgium), carbidopa (Merck, Sharp and Dohme, Harlow) and 5-HTP (Sigma Chemical Company, Poole) were given as suspensions in 0.005% BRIJ (polyoxylethylene laurylester) in 0.9% NaCl. Antagonists were injected s.c. and other drugs i.p. (mCPP, carbidopa, 5-HTP) in a volume of 1 ml kg^{-1} body weight with the exception of 5-HTP which was given in 2 ml kg^{-1} body weight.

Statistics

The effects of antagonists and mCPP alone on food intake were analysed by Dunnett's test following significant one-way ANOVA. ID_{50} values were calculated from the ranges of antagonist doses producing 20–80% inhibition of either the head shakes or the hypophagic effect of mCPP by least squares linear regression, except in the case of ritanserin when the ID_{50} was calculated by interpolation between the two values in this range before maximum inhibition was attained (see Figure 1a). Two tailed 95% confidence limits of the values were calculated by the method of Bowman & Rand (1980). ID_{50} values were correlated with published affinities (Hoyer, 1988; Schoeffter *et al.*, 1988; Schoeffter & Hoyer, 1989; Schlicker *et al.*, 1989) following log transformation to minimize spurious significance due to the skewed distribution of values (Winer, 1971). Ratios of ID_{50} values were also log-transformed before correlation testing.

Results

Effects of 5-hydroxytryptamine antagonists on 1-3(chlorophenyl)piperazine-induced hypophagia

Groups of food-deprived rats dosed subcutaneously with 5 mg kg^{-1} (18.4 μmol kg^{-1}) mCPP 20 min before restoration of food consumed 51–79% less food over the next 2 h than control rats dosed subcutaneously with 0.9% NaCl (Table 1). 5-HT antagonists (with the exception of ritanserin) caused significant overall dose-dependent inhibition of mCPP-induced hypophagia (Figure 1a). Ritanserin had a partial effect causing about 58% inhibition at 5 mg kg^{-1} (10.4 μmol kg^{-1}). Higher doses up to 40 mg kg^{-1} (83 μmol kg^{-1}) produced no further inhibition. None of the antagonists significantly affected food intake of food-deprived rats in the absence of mCPP even at the highest dose used (Table 1). Calculated ID_{50} values (including that of ritanserin) are given in Table 2 and were in order: metergoline < pizotifen < 1-NP < mianserin < ritanserin < methysergide < LY 53587 < altanserin < ketanserin < (±)-propranolol. Figure 2a shows the significant relationship between these values and published *in vitro* affinities (pK_D) of the drugs for the 5-HT$_{1C}$ receptor subtype (given in Table 2). Corresponding correlations with affinities for 5-HT$_{1A}$, 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes and for the 5-HT$_2$ receptor (Table 2) were not significant.

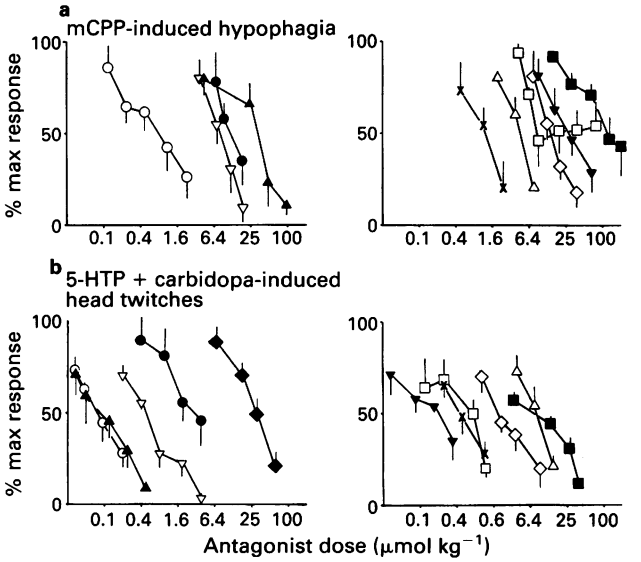

Figure 1 (a) Effects of various doses of antagonists on 1-3(chlorophenyl)piperazine (mCPP, 5 mg kg^{-1}, i.p.)-induced hypophagia in 18 h food-deprived rats. Antagonists were injected s.c. 20 min prior to mCPP and 40 min prior to food restoration. Results are shown in % form with 100% representing the decrease of food intake over 2 h in the absence of antagonists (means with s.e.mean shown by vertical lines, n = 5–7 for each drug concentration). Significance of correlations between antagonist dose and response are shown in parentheses as follows: metergoline (○) ($P < 0.01$), 1-naphthyl piperazine (△) ($P < 0.001$), mianserin (▽) ($P < 0.02$), ritanserin (□) (NS), methysergide (◇) ($P < 0.05$), LY 53857 (●) ($P < 0.05$), altanserin (▲) ($P < 0.01$), ketanserin (▼) ($P < 0.05$), (±)-propranolol (■) ($P < 0.01$), pizotifen (×) ($P < 0.05$). (b) Effects of various doses of antagonists on carbidopa (25 mg kg^{-1}, i.p.) + 5-hydroxytryptophan (5-HTP, 100 mg kg^{-1}, i.p.)-induced head shakes. The antagonists were injected s.c. immediately after carbidopa, 30 min before 5-HTP injection and 1 h before the start of the scoring period. Results are shown in % form as means with s.e.mean shown by vertical lines (n = 5–7 for each drug concentration) with 100% representing the number of head twitches in the absence of antagonists. This varied from experiment to experiment between 14 and 30. Significance of correlations between antagonist dose and response are shown in parentheses as follows: metergoline (○) ($P < 0.01$), 1-naphthyl piperazine (△) ($P < 0.001$), mianserin (▽) ($P < 0.01$), ritanserin (□) ($P < 0.05$), methysergide (◇) ($P < 0.02$), LY 53857 (●) (NS), altanserin (▲) ($P < 0.05$), ketanserin (▼) ($P < 0.05$), (±)-propranolol (■) ($P < 0.01$), pizotifen (×) ($P < 0.05$), mCPP (◆) ($P < 0.001$).

Effects of 5-hydroxytryptamine antagonists on head shakes

Results in Figure 1b show that all 10 antagonists and also mCPP inhibited head shakes induced by 25 mg kg^{-1} car-

Table 1 Effects of 1-3(chlorophenyl)piperazine (mCPP) and of 5-hydroxytryptamine (5-HT) antagonists at high dosage on 2 h food intake of 20–21 h food-deprived rats

Treatment	*Food intake* ($g\,2h^{-1}$)
0.9% NaCl (8)	8.0 ± 0.6
Altanserin 71.1 $\mu mol\,kg^{-1}$ (4)	6.5 ± 0.6
mCPP 18.4 $\mu mol\,kg^{-1}$ (8)	2.1 ± 0.5*
0.9% NaCl (8)	8.3 ± 1.8
Ketanserin 50 $\mu mol\,kg^{-1}$ (6)	7.5 ± 1.9
mCPP 18.4 $\mu mol\,kg^{-1}$ (9)	2.7 ± 0.7*
0.9% NaCl (10)	6.9 ± 0.5
LY 53857 20 $\mu mol\,kg^{-1}$ (5)	6.2 ± 0.6
mCPP 18.4 $\mu mol\,kg^{-1}$ (9)	3.4 ± 0.4*
0.9% NaCl (10)	8.3 ± 0.6
Metergoline 2.5 $\mu mol\,kg^{-1}$ (6)	7.1 ± 0.9
mCPP 18.4 $\mu mol\,kg^{-1}$ (6)	3.1 ± 0.7*
0.9% NaCl (7)	6.4 ± 0.4
Methysergide 84.6 $\mu mol\,kg^{-1}$ (4)	5.0 ± 0.6
mCPP 18.4 $\mu mol\,kg^{-1}$ (10)	3.0 ± 0.8*
0.9% NaCl (13)	5.8 ± 0.4
Mianserin 20 $\mu mol\,kg^{-1}$ (7)	6.2 ± 0.4
0.9% NaCl (10)	7.3 ± 0.7
1-NP 8.2 $\mu mol\,kg^{-1}$ (8)	6.8 ± 1.5
mCPP 18.4 $\mu mol\,kg^{-1}$ (10)	2.7 ± 1.0*
0.9% NaCl (8)	8.1 ± 1.1
(±)-Propranolol 174 $\mu mol\,kg^{-1}$ (4)	6.1 ± 1.5
mCPP 18.4 $\mu mol\,kg^{-1}$ (8)	1.7 ± 0.6*
0.9% NaCl (8)	8.2 ± 1.8
Ritanserin 80 $\mu mol\,kg^{-1}$ (5)	6.4 ± 1.3
mCPP 18.4 $\mu mol\,kg^{-1}$ (7)	2.7 ± 0.7*
0.9% NaCl (16)	8.4 ± 1.8
Pizotifen 5 $\mu mol\,kg^{-1}$ (7)	7.3 ± 2.2
mCPP 18.4 $\mu mol\,kg^{-1}$ (10)	3.5 ± 2.0*

Results are shown as means ± s.e.mean with numbers of rats in parentheses. * $P < 0.01$ vs appropriate 0.9% NaCl-treated group by Dunnett's test following significant 1-way ANOVA. 1-NP = 1-naphthyl piperazine.

bidopa and 100 $mg\,kg^{-1}$ 5-HTP. Significant regressions were obtained between dose of antagonist and corresponding response inhibition (Figure 1b) with the exception of LY 53857. None of the antagonists caused head shakes when given alone. Calculated ID_{50} values are given in Table 2 and were in order: metergoline = ketanserin < altanserin < ritanserin < mianserin < pizotifen < methysergide = LY 53857 < (±)-propranolol = 1-NP < mCPP. Figure 2b shows that the correlation between these values and the *in vitro* affinities (pK_D) of the drugs for the 5-HT_2 receptor (given in Table 2) was significant ($P < 0.01$) but that corresponding correlations with affinities for 5-HT_1 receptor subtypes (Table 2) were not significant.

Comparison of effects of antagonists on 1-3(chlorophenyl)piperazine-induced hypophagia and head shakes

ID_{50} values against mCPP-induced hypophagia did not correlate significantly with ID_{50} values against head shakes ($r = 0.40$, d.f. 8, NS).

Inspection of Figure 2a and b reveals that ID_{50}s for inhibition of hypophagia and head shakes correlated significantly with affinities (pK_D) for 5-HT_{1C} and 5-HT_2 sites respectively. However, ID_{50} values for methysergide and ritanserin were rather larger than predictable from the overall relationships between ID_{50} and affinity (approximately 3 fold for hypophagia in both cases and 6 fold (methysergide) and 3 fold (ritanserin) for head shakes). These common discrepancies from linearity largely cancel each other out when ratios of the two ID_{50} values are plotted against the corresponding ratio of affinities (Figure 2c) so that the correlation between the ratios is more significant than that between ID_{50} values against hypophagia and affinities for 5-HT_{1C} sites. Significant correlations were not obtained when the ratios of the ID_{50}s were plotted against the ratios of the affinities for the 5-HT_{1A}, 5-HT_{1B} or 5-HT_{1D} sites to the affinities for 5-HT_2 sites.

Discussion

The present findings strengthen previous evidence (Kennett & Curzon, 1988a) that mCPP causes hypophagia by activating 5-HT_{1C} receptors. Thus, ID_{50} values for inhibition of the hypophagia by 5-HT antagonists correlated significantly with published *in vitro* 5-HT_{1C} receptor affinities but not with affinities for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} or 5-HT_2 receptors. Affinities for 5-HT_2 receptors correlated significantly with ID_{50}s for inhibition of 5-HTP-induced head shakes, which depend on activation of 5-HT_2 sites (Bedard & Pycock, 1977; Yap & Taylor, 1983; Niemegeers *et al.*, 1983).

When we began this study we expected that some antagonists would show a relatively week relationship between ID_{50} against mCPP-induced hypophagia and affinity for 5-HT_{1C}

Table 2 ID_{50} values for inhibition of 1-3(chlorophenyl)piperazine (mCPP)-induced hypophagia and carbidopa and 5-hydroxytryptophan (5-HTP)-induced head shakes and corresponding affinities for 5-HT receptor subtypes

Drug	ID_{50} ($\mu mol\,kg^{-1}$) *vs hypophagia*	ID_{50} ($\mu mol\,kg^{-1}$) *vs head shakes*	$\frac{ID_{50}\ (hypophagia)}{ID_{50}\ (head\ shakes)}$	*Affinity* ($-\log M^{-1}$) 5-HT_{1A}	5-HT_{1B}	5-HT_{1C}	5-HT_{1D}	5-HT_2
Metergoline	0.50 (0.30–0.94)	0.084 (0.025–2.5)	5.9	8.10	7.39	9.19	9.09	9.03
Pizotifen	1.11 (0.45–2.65)	1.25 (0.52–2.71)	0.89	6.10	5.50	8.1	5.65	7.8
1-NP	4.1 (2.5–5.7)	7.06 (6.4–7.9)	0.57	7.18	6.56	8.24	7.83	7.24
Mianserin	8.4 (4.8–13.6)	0.48 (0.4–0.6)	17.5	6.03	5.21	8.00	6.37	8.08
Ritanserin	9.6 (3.8–24.4)	0.40 (0.19–0.79)	24.4	5.37	4.00	8.64		9.25
Methysergide	11.0 (7.4–16.0)	2.30 (1.2–4.5)	4.82	7.63	5.82	8.61	8.42	8.57
LY 53857	12.8 (6.6–24.6)	2.40 (1.2–4.5)	5.42	6.41	5.53	8.08		7.34
Altanserin	24.9 (18.5–31.6)	0.144 (0.075–0.28)	173	5.55	5.98	6.93		8.58
Ketanserin	32.2 (22.2–47.2)	0.09 (0.045–0.18)	358	5.86	5.72	7.01	6.00	8.86
(±)-Propranolol	142.0 (101.0–181.7)	6.7 (4.8–9.4)	21.2	6.48	7.07	6.23	5.39	6.46

95% confidence limits of ID_{50} values are shown in parentheses. Affinity data from Hoyer (1988), Schoeffter & Hoyer (1989), Schoeffter *et al.* (1988) and Schlicker *et al.* (1989) using membranes from pig brain cortex (5-HT_{1A}), rat brain cortex (5-HT_{1B}), pig choroid plexus (5-HT_{1C}), pig caudate nucleus (5-HT_{1D}) and rat brain cortex (5-HT_2).
The ID_{50} values can be converted from $\mu mol\,kg^{-1}$ to $mg\,kg^{-1}$ by multiplying by the following: metergoline 0.403, pizotifen 0.395, 1-NP 0.249, mianserin 0.251, ritanserin 0.478, methysergide 0.469, LY 53857 0.501, altanserin 0.562, ketanserin 0.409, (±)-propranolol 0.296, mCPP 0.270.

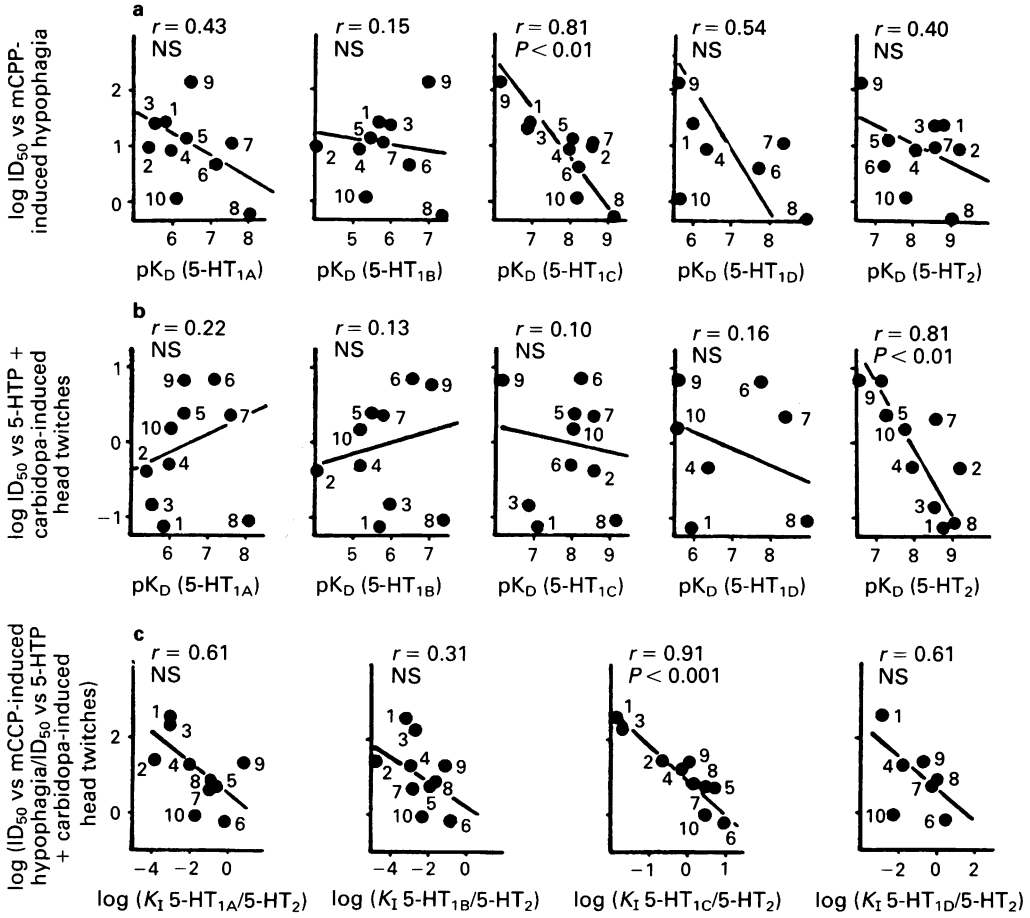

Figure 2 (a) Correlations between log [ID_{50} (μmol kg^{-1}) vs 1-3(chlorophenyl)piperazine (mCPP)-induced hypophagia] and $-$log affinity (pK_D) for 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, 5-HT$_{1D}$ or 5-HT$_2$ receptors (data from Table 2). The following 10 antagonists were used: (1) ketanserin, (2) ritanserin, (3) altanserin, (4) mianserin, (5) LY 53857, (6) 1-naphthyl piperazine, (7) methysergide, (8) metergoline, (9) ($\pm$)-propranolol and (10) pizotifen. Correlation coefficients (r) and statistical significances of correlations are given. NS = not significant. (b) Correlations between log [ID_{50} (μmol kg^{-1}) vs 5-hydroxytryptophan (5-HTP) + carbidopa-induced head shakes] and $-$log affinity (pK_D) for 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, 5-HT$_{1D}$ and 5-HT$_2$ receptors for each of 10 antagonists (key as in (a)). (c) Correlations between log [ID_{50} vs mCPP-induced hypophagia/ID_{50} 5-HTP + carbidopa-induced head twitches] and logs of the following ratios of affinities (K_I) for receptors: 5-HT$_{1A}$/5-HT$_2$, 5-HT$_{1B}$/5-HT$_2$, 5-HT$_{1C}$/5-HT$_2$ or 5-HT$_{1D}$/5-HT$_2$. Values for each of the 10 antagonists in (a) and (b). (Key as in (a)).

sites. This was found for ritanserin, methysergide and pizotifen. Rather similar deviations were found for the first two drugs in the relationship between ID_{50} against 5-HTP-induced head shakes and affinity for 5-HT$_2$ sites. These common deviations presumably reflect aspects of the metabolism or disposition of the drugs which comparably affect their availability to both central 5-HT$_{1C}$ and 5-HT$_2$ sites *in vivo* but not *in vitro*. They therefore tend to cancel out when the ratios of ID_{50} values for inhibition of the two behavioural effects are calculated. Thus, the ratios correlate more significantly with the corresponding ratios of 5-HT$_2$ to 5-HT$_{1C}$ receptor affinities than do the respective ID_{50} values with the affinities for the individual sites. This further strengthens the hypothesis that mCPP-induced hypophagia is mediated by 5-HT$_{1C}$ receptor activation. A somewhat similar procedure has been used by Leysen *et al.* (1978) when studying the binding of neuroleptics at 5-HT receptors.

The inability of ritanserin to antagonize completely mCPP-induced hypophagia was striking; rather similar but less detailed findings are reported for the inhibition of quipazine-induced hypophagia by both ritanserin and ketanserin (Hewson *et al.*, 1989) and for the inhibition of fenfluramine-induced hypophagia by ritanserin (Neill & Cooper, 1989). Partial inhibition could conceivably be due to the ritanserin being a partial agonist but this seems unlikely as it did not cause hypophagia when given alone. Another possibility which cannot be excluded is that (uniquely among the antagonists tested) it discriminates between two sites which are simultaneously stimulated by mCPP to induce hypophagia.

In agreement with Simansky & Schechter (1987), mCPP blocked 5-HT-induced head shakes. It therefore appears to be not an agonist but an antagonist at 5-HT$_2$ sites. This is consistent with its inhibitory effect on the stimulation of cortical phosphoinositide hydrolysis by 5-HT (Conn & Sanders Bush, 1987).

In the present study, ketanserin and ritanserin inhibited mCPP-induced hypophagia with ID_{50} values of 32.2 and 9.6 μmol kg^{-1}. The failure of Aulakh *et al.* (1989) to antagonize the hypophagia with 10.4 μmol kg^{-1} ritanserin (albeit under unspecified conditions) is therefore surprising. The finding of Klodzinska & Chojnacka-Wojcik (1990) that TFMPP-induced hypophagia was opposed by 6.2 and 1.05 μmol kg^{-1} respectively of ketanserin and ritanserin may reflect their use of freely feeding rats as, under these conditions, feeding is significantly more sensitive to the hypophagic effects of mCPP (and RU 24969) and hence possibly also to their antagonists, than when food-deprived animals are used as in the present study (Table 3). A similar argument may also explain why doses of ketanserin and ritanserin needed to antagonize hypophagias induced by fenfluramine (Hewson *et al.*, 1988), quipazine (Hewson *et al.*, 1989) and DOI (Schechter & Simansky, 1988) were less than those we found to inhibit

Table 3 Calculated ED_{50} values (and 95% confidence limits) for the hypophagic response to 1-3(chlorophenyl)piperazine (mCPP) and RU 24969 in freely feeding and food-deprived rats

	ED_{50} (μmol kg^{-1})	
Drug	*Freely feeding*	*Food-derived*
mCPP	2.56 (1.04–5.9)	10.3 (8.4–12.5)*
RU24969	2.37 (1.1–5.2)	16.3 (9.2–32.3)*

Significant difference * $P < 0.001$ by t test following comparison of significant dose-response regression lines using data from Kennett *et al.* (1987) and Kennett & Curzon (1988a) expressed as percentages and Arcsin-transformed (Winer, 1971) as outlined by Bowman & Rand (1980).

mCPP-induced hypophagia as these groups used fed rats exposed to a palatable diet.

Most of these findings by other groups were taken to suggest that the hypophagic effects reported resulted from activation of 5-HT_2 receptors. However, the above comments and the present findings point strongly to the involvement of 5-HT_{1C} sites. It is also relevant that the doses of ketanserin and ritanserin required for inhibition of hypophagia were much greater than those we found necessary for inhibition of 5-HT_2 receptor-mediated head shakes. Furthermore, Neill & Cooper (1989) did not confirm the inhibitory effect of ketanserin on fenfluramine-induced hypophagia reported by Hewson *et al.* (1988).

The present findings strengthen our previous evidence (Kennett & Curzon, 1988a,b) that mCPP-induced hypophagia occurs through the activation of 5-HT_{1C} receptors. Our work also suggested that 5-HT_{1B} receptors were needed for the response to occur in the rat (Kennett & Curzon, 1988a). As 5-HT_{1D} receptors have a similar distribution to rat 5-HT_{1B} receptors in species in which these are absent (man, pig, calf) (Waeber *et al.*, 1988) a similar requirement for 5-HT_{1D} sites may occur in the latter species.

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Barbiturates inhibit ATP-K^+ channels and voltage-activated currents in CRI-G1 insulin-secreting cells

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1 Patch-clamp recording techniques were used to examine the effects of barbiturates upon the ATP-K^+ channel, and voltage-activated channels present in the plasma membrane of CRI-G1 insulin-secreting cells.

2 Thiopentone inhibited ATP-K^+ channel activity when applied to cell-attached patches or the intracellular or extracellular surface of cell-free patches. Secobarbitone and pentobarbitone were also effective inhibitors of ATP-K^+ channels in cell-free patches, whereas phenobarbitone was ineffective.

3 The diabetogenic agent, alloxan, which is structurally related to the barbiturates also produced an inhibition of ATP-K^+ channel activity in outside-out patches.

4 Whole-cell ATP-K^+ currents were used to quantify the effects of the barbiturates: concentration-inhibition curves for thiopentone, secobarbitone and pentobarbitone resulted in IC_{50} values of 62, 250 and 360 μM respectively. Phenobarbitone at a concentration of 1 mM was virtually ineffective.

5 Calculation of the apparent membrane concentrations for these drugs indicate that for a given degree of ATP-K^+ channel inhibition a similar concentration of each barbiturate is present in the membrane. This suggests that hydrophobicity plays a primary role in their mechanism of action. The pH-dependence and additive nature of barbiturate block also indicates a membrane site of action.

6 Thiopentone, (100 μM) was also found to inhibit differentially voltage-activated whole-cell currents. The relative potency of thiopentone at this concentration was 0.64, 0.38 and 0.12 for inhibiting Ca^{2+}, K^+ and Na^+ currents respectively when compared with its ability to inhibit the ATP-K^+ channel.

Keywords: Barbiturate; ATP-K^+ channels; Ca^{2+} currents; Na^+ currents; K^+ currents; β-cells

Introduction

Barbiturates elicit a bewildering number of actions upon neuronal tissue including the reduction of excitability by inhibition of voltage-dependent currents. For example they reduce the duration of calcium-dependent action potentials (Goldring & Blaustein, 1982; Heyer & MacDonald, 1982) and inhibit voltage-dependent calcium currents in invertebrate (Nishi & Oyama, 1983; Ikemoto *et al.*, 1986) and vertebrate neurones (Gross & Macdonald, 1988). Pentobarbitone has been reported to reduce the amplitude of voltage-activated sodium and potassium conductances (Blaustein, 1968; Narahashi *et al.*, 1971; Sevcik, 1980; Zbicz & Wilson, 1981), probably via the uncharged form of the molecule (Blaustein, 1968; Narahashi *et al.*, 1971; Zbicz & Wilson, 1981). In addition Blaustein (1968) and Sevcik (1980) have shown that thiopentone reduces the potassium permeability in axons of lobster and squid.

The ability of this group of compounds to affect ion transport may also be important with respect to β-cell function, insulin secretion and plasma glucose levels *in vivo*. However, conflicting results have arisen from both *in vivo* and *in vitro* studies. For example, pentobarbitone has been reported to decrease insulin levels *in vivo* after glucose load, in dogs (Renauld & Sverdlik, 1975), and in rats (Aynsley-Green *et al.*, 1973) and to reduce insulin release from mouse pancreatic β-cells *in vitro* (Panten *et al.*, 1973; Hellman, 1977). However, Aynsley-Green *et al.* (1973) have observed that intravenous pentobarbitone does not affect blood glucose in starved rats, and Mennear *et al.* (1976) found that pentobarbitone has no effect on blood glucose concentration during intravenous glucose tolerance tests in mice.

More recently, thiopentone has been shown (Gonçalves *et al.*, 1986) to depolarize mouse β-cell membrane potential in the absence of extracellular glucose and to increase insulin release from rat isolated islets in the presence of 6 mM glucose (equivalent to fasting level). These actions are associated with an increased mouse β-cell input resistance and a reduced $^{86}Rb^+$ efflux rate from rat islets indicating that thiopentone inhibits the potassium permeability of islet β-cells. However, thiopentone was also reported to have little effect on $^{86}Rb^+$ efflux in the presence of a high (16.7 mM) concentration of glucose. These actions of thiopentone are very similar to those of the sulphonylurea group of drugs which elicit insulin release by inhibition of ATP-sensitive K^+ (ATP-K^+) channels (Sturgess *et al.*, 1985; Ashford, 1990). Although the overall effect of barbiturates on insulin release is not clear from these studies, in general, inhibition is observed in already stimulated β-cells (after glucose load) where ATP-K^+ channels are mostly closed (and the cell is depolarized). This is in contrast to the secretory action of thiopentone at low (fasting) levels of glucose where ATP-K^+ channels are more active (and the cell has a more negative membrane potential). Thus it is possible that the barbiturates have more than one action on pancreatic β-cell ion currents, causing inhibition of ATP-K^+ channels, depolarization and insulin release in non-stimulated cells and inhibition of insulin release via some other mechanism in stimulated cells. In view of these observations we have investigated the effects of thiopentone on ATP-K^+ channel currents and voltage-activated conductances in CRI-G1 cells, which we have previously shown to be a good electrophysiological model for pancreatic β-cells (Sturgess *et al.*, 1986; Kozlowski *et al.*, 1991). We have also compared the effects of thiopentone on ATP-K^+ channel currents with three other barbiturates: secobarbitone, phenobarbitone and pentobarbitone. In addition we have examined the actions of alloxan, structurally a closely related compound to the barbiturates. This compound produces a diabetogenic action in man and most other animals via islet β-cell destruction. It induces a bi- or triphasic blood glucose response dependent upon the fasted state of the animal (Rerup, 1970). Although its mechanisms of action are not understood, alloxan has also been reported to depolarize

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Table 1 Composition of solutions used in experiments (in mM)

Solution	*NaCl*	*KCl*	*CsCl*	*NMDG*	*HCl*	*$CaCl_2$*	*$MgCl_2$*	*ATP*	*EGTA*	*pH*
A	135	5	—	—	—	1	1	—	—	7.4/7.2
B	—	140	—	—	—	5	5	—	—	7.2
C	—	140	—	—	—	1	1	—	—	7.2
D	—	140	—	—	—	4.6	—	—	10	7.2
E	—	140	—	—	—	—	—	—	1	7.2
F	—	140	—	—	—	2	1.1	0.1	10	7.2
G	135	5	—	—	—	1	1	—	—	7.4/9.0
H	—	140	—	—	—	2	2.5	2	10	7.2
I	—	—	140	—	—	2	2.5	2	10	7.2
J	—	—	—	140	140	2	2.5	2	10	7.2

HEPES (10 mM) was present in all solutions. NMDG is N-methyl-D-glucamine.

pancreatic β-cells through an interaction with a membrane component (Dean & Matthews, 1972). Some of the results presented here have already appeared in abstract form (Ashford *et al.*, 1989; Kozlowski *et al.*, 1989a).

Methods

Cell culture

Cells of the rat pancreatic islet cell-line (CRI-G1) were cultured and passaged at 4–7 day intervals as previously described (Carrington *et al.*, 1986). Cells used for patch-clamp experimentation were plated onto 3.5 cm petri dishes (Sterilin), at a density of approximately 1.5×10^5 cells per dish. The cells were used 2 to 6 days inclusive after plating.

Electrical recording and analysis

Ionic currents (single channel and whole cell) were detected with a patch-clamp amplifier (List EPC-7) and were recorded onto magnetic tape with a bandwidth (3 db down) of 1250 Hz (Racal 4D Tape recorder) for off-line analysis at a later date. All experiments were conducted at room temperature (20–23°C).

Isolated patch studies Single channel currents were recorded from cell-attached, outside-out and inside-out membrane patches by standard patch clamp recording procedures (Hamill *et al.*, 1981). Recording electrodes, when filled with electrolyte, had resistances between 8–15 MΩ. Single channel records used for illustrative purposes were replayed into a chart recorder (Gould 2200) which filtered the data at ~140 Hz. The potential across the membrane patch is described following the usual sign convention for membrane potential (i.e. inside negative). Outward current (defined as current flowing from the inside to the extracellular side of the membrane) is shown as upward deflections on all traces. Single channel current analysis was carried out off-line by use of a programme that incorporates a 50% threshold crossing parameter to detect events (Dempster, 1988) and run on an Apricot XEN-Xi286/45 microcomputer. Data segments between 30 and 120 s were replayed at the recorded speed and filtered at 1 kHz with an 8 pole bessel filter and digitized at 5 kHz with a Data Translation 2801A interface. The average channel activity ($N_f \cdot P_o$), where N_f is the number of functional channels in the patch and P_o is the open-state probability, was determined by measuring the total time spent at each unitary current level and expressed as a proportion of the total time recorded (Kozlowski *et al.*, 1989b).

Whole-cell studies Whole-cell ionic currents were recorded from single CRI-G1 cells by standard whole-cell voltage clamp procedures (Hamill *et al.*, 1981). Electrodes had resistances between 1–6 MΩ in these experiments. To obtain whole-cell ATP-K^+ currents, the cell was voltage-clamped at a holding potential of −70 mV, and alternate ±10 mV pulses, 200 ms in duration were applied every 2 s (see also Trube *et al.*, 1986; Kozlowski *et al.*, 1989b). Under these conditions (no ATP in the electrode), dialysis of the cell interior with pipette solution (i.e. washout of the intracellular ATP) results in an increase in the overall potassium conductance and this is due to the activation of the ATP-sensitive K^+ channel (Rorsman & Trube, 1985; Sturgess *et al.*, 1988). Drug effects were quantified by measuring the amplitude of the current responses (I_B) during drug exposure and comparing them with those recorded under control conditions (I_C) immediately preceding drug administration. These data were used to construct concentration-response relationships which were fitted either by eye or to the Hill equation according to Treherne & Young (1988).

Whole-cell, voltage-activated currents were evoked by use of 'VGEN', a programme designed to produce a series of voltage-clamp command pulse patterns (Dempster, 1988), and run on an Apricot microcomputer with a DT 2801A interface. Inward Na^+ currents were evoked by applying depolarizing voltage pulses, 15 ms duration, at a frequency of 1 Hz. Delayed outward currents and inward calcium currents were generated in response to voltage steps, 100 ms in duration, at the same frequency. The frequency of stimulation did not affect the amplitude of the currents. Voltage-activated currents were analysed by 'VCAN', a whole-cell clamp analysis package (Dempster, 1988). Currents were replayed at the recorded speed into the computer through the DT 2801A interface. Two digitization rates were used, 330 μs per point for voltage pulses 100 ms in duration, and 80 μs per point for pulses 15 ms in duration. Data were then designated either a test or leak signal and as each voltage step was repeated in triplicate or quadruplicate, an average file was created in order to improve the signal to noise ratio. After signal averaging, leak subtraction was performed (leak current responses were acquired by evoking a current in response to a 20 mV hyperpolarizing pulse). Current-voltage plots were constructed by use of 'VCAN' and all currents were measured relative to the current level preceding application of the test pulse. For illustrative purposes these records were plotted with a digital plotter (Hewlett Packard HP7475A).

No arithmetic compensation was made for ATP-K^+ channel rundown, as described by Trube *et al.* (1986) and Sturgess *et al.* (1988) since experiments were conducted (whole-cell and single channel) under conditions where rundown was virtually eliminated (Kozlowski & Ashford, 1990). All data in text and figures are presented as mean values ± s.e.mean unless otherwise stated. The statistical significance between experimental groups was assessed by Student's *t* test for unpaired data.

Solutions

Isolated patch studies Before use, cells were washed thoroughly with normal bath saline (solution A, see Table 1) and this solution was also used to bathe the cells during cell-attached recording whilst the recording pipette contained a

high K^+-containing saline (solution B). For isolated patch studies the extracellular surface was bathed in solution A or a potassium rich solution (C) whilst the intracellular surface was bathed with solution D which contained free Ca^{2+} and Mg^{2+} concentrations of $\sim$25 nM and $<$5 nM respectively. In some experiments solution D was replaced with solution E which resulted in free Ca^{2+} and Mg^{2+} concentrations of $<$2 nM and 4 μM respectively.

Whole-cell studies For all whole-cell recordings cells were bathed in solution A whilst the cell interior was dialysed with solution D, or solution F which contained free Ca^{2+} and Mg^{2+} concentrations of $\sim$25 nM and 0.65 mM respectively. Whole cell experiments comparing drug effects at different pH values used a slightly modified extracellular solution (solution G) which contained Tris buffer since HEPES is an inadequate buffer at pH 9.0. Conditions and solutions for evoking whole-cell voltage activated currents were identical to those we have previously described (Kozlowski *et al.*, 1991). To isolate delayed outward currents cells were bathed in solution A to which $CdCl_2$ (1 mM) and tetrodotoxin (TTX, 300 nM) had been added and a potassium-rich saline (solution H) containing 2 mM ATP (to reduce ATP-K^+ current) dialysed the cell interior. For isolation of sodium currents, cells were bathed in normal physiological saline (solution A), which also contained 1 mM Cd^{2+} to block inward Ca^{2+} currents and 20 mM tetraethylammonium (TEA) and 4 mM 4-aminopyridine (4-AP) to block any outward current through K^+ channels and were dialysed with solution I which contained Cs^+ ions and ATP to block outward currents. To examine the effects of thiopentone on Ca^{2+} currents, solution J dialysed the interior of whole-cells and this contained 140 mM N-methyl-D-glucamine (NMDG), which replaced the K^+ contained in solution H, as the predominant cation. In addition, TTX (300 nM) was included in the bathing medium (solution A), to block the Na^+ current, and the concentration of Ca^{2+} increased to 10 mM to enhance the size of the inward Ca^{2+} current. The concentration of Ca^{2+} and Mg^{2+} in solutions H, I and J was maintained at $\sim$20 nM and 0.5 mM respectively. The concentration of free divalent cations was determined by use of 'METLIG', a programme for calculating metal ion/ligand binding (England, P. & Denton, R.; University of Bristol).

The barbiturates thiopentone, pentobarbitone, phenobarbitone and secobarbitone, were prepared daily as stock solutions (0.1 M) with NaOH (0.1 M) as solvent. Alloxan was dissolved directly in the electrolyte used in the experiment at the concentration required, and protected from breakdown by ultra violet light by wrapping in silver foil. ATP was prepared as a stock solution in distilled H_2O at concentrations ranging from 15 to 30 mM and stored frozen in small aliquots at -20°C. Any significant changes in pH or ionic concentration by addition of the drug and its vehicle were compensated by adjusting the pH or adding the equivalent concentration of vehicle to the drug-free saline used throughout the experiments. Solutions were exchanged during recordings by superfusion of the whole bath with a gravity feed system; expiration was by suction with a vacuum pump (HyFlo Ltd.). Approximately, 20–25 ml of solution were required for complete solution exchange, which took approximately 45 s.

Drugs

The chemicals used were: ATP (adenosine-5'-triphosphate, dipotassium and disodium salts), alloxan, 4-aminopyridine (4-AP), ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-*bis*-(b-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA), (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]) (HEPES), pentobarbitone (sodium salt), phenobarbitone, secobarbitone (sodium salt), tetraethylammonium chloride (TEA), tetrodotoxin (TTX), thiopentone (sodium salt/sodium carbonate mixture), Tris, Trizma base and Trizma hydrochloride and were all obtained from Sigma (Poole, Dorset). The $\log_{10}$ P values for the barbiturates were obtained from Leo *et al.* (1971) and the pK_a values were obtained from the following sources: thiopentone (Dundee, 1974) secobarbitone, phenobarbitone and pentobarbitone (Harrison & Simmonds, 1983).

Results

Single ATP-K^+ channel studies

To determine whether thiopentone inhibited ATP-K^+ channels in CRI-G1 insulin-secreting cells in the absence of glucose or other metabolizable substrates, the cell-attached configuration was utilized. Cells were bathed in solution A whilst solution B, containing 140 mM KCl, was present in the pipette. Application of thiopentone (300 μM) to the bath induced a marked and reversible decrease in ATP-K^+ channel activity ($n = 2$) as shown in Figure 1a, which depolarized the β-cell, and decreased the driving force for K^+ ions into the cell. This latter action also induced a reduction in the amplitude of single channel events from 4.1 pA in control to 3.4 pA in the presence of the drug, which was reversed on wash (to 4.1 pA). These results indicate that thiopentone does inhibit ATP-K^+ channels, but does not resolve whether this is mediated by a direct or indirect interaction with the channels. Consequently, the effects of thiopentone and three other barbiturates, pentobarbitone, phenobarbitone and secobarbitone were examined upon ATP-K^+ channels recorded from isolated membrane patches. For both the inside-out and outside-out patch configurations solution C bathed the extracellular membrane surface, whilst solution D containing a low divalent cation concentration bathed the intracellular aspect of the patch.

Application of thiopentone (300 μM) to the extracellular surface of outside-out membrane patches (Figure 1b), resulted in almost complete inhibition of outward ATP-K^+ channel currents ($n = 5$). Both the on-rate and off-rate were rapid, with full reversal occurring upon washout of the drug. Similar data were obtained with inside-out membrane patches where the drug was applied to the intracellular aspect of the membrane ($n = 3$). The oxygen-substituted analogue of thiopentone, pentobarbitone (300 μM), also reversibly decreased ATP-K^+ channel activity ($n = 6$) when applied to either membrane surface but was much less effective than thiopentone at the same concentration (data not shown). Similarly, secobarbitone (300 μM) when applied to the external aspect of outside-out membrane patches induced a marked degree of channel inhibition ($n = 5$) which was also reversible. In contrast, phenobarbitone (300 μM) was virtually ineffective (Figure 1c) when applied to the extracellular membrane surface of outside-out patches ($n = 6$), or the intracellular aspect of inside-out membrane patches ($n = 4$). The inhibitory effects of all four barbiturates were tested at both hyperpolarized and depolarized membrane potentials and were found to be independent of membrane polarity. Alloxan, a compound structurally related to the barbiturates, induced a partially reversible ATP-K^+ channel inhibition ($n = 2$) when applied to the extracellular membrane surface of outside-out patches, but only at high (mM) concentrations (Figure 1d).

Barbiturate effects on whole-cell ATP-K^+ currents

The results described above clearly show that ATP-K^+ channel activity is inhibited by a range of barbiturates. In order to quantify these effects, the whole-cell voltage-clamp recording configuration was used and ATP-K^+ currents were evoked with the twin pulse protocol described in the Methods. The whole-cell dialysing solution (solution D) was identical to that used to bathe the intracellular aspect of isolated membrane patches described above.

A significant inhibition of whole-cell ATP-K^+ currents by thiopentone was observed at concentrations greater than 10 μM and the inhibitory effect became progressively greater with increasing concentration of the barbiturate until at 1 mM, thiopentone virtually abolished the current. The inhibition of the ATP-K^+ current by thiopentone was readily reversed

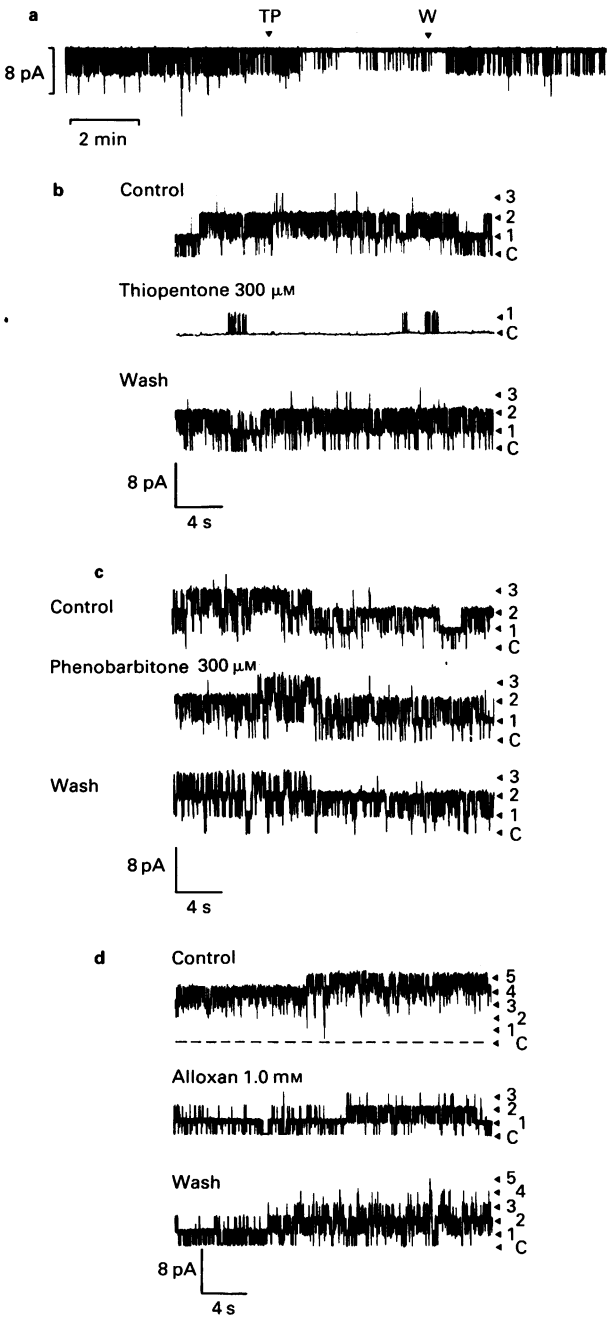

Figure 1 (a) Effects of thiopentone upon ATP-K^+ channels recorded in the cell-attached configuration. The cell was bathed in normal physiological saline (solution A) whilst the pipette contained solution B. The membrane patch was voltage-clamped at a holding potential of 0 mV. Prior to addition of thiopentone (TP) a maximum of three single channel openings occurred simultaneously, these are indicated by downward deflections of the trace. Application of 300 μM thiopentone caused a reduction in channel activity, that was partly reversible upon washout of the drug. Values of $N_f \cdot P_o$ are: control 0.078; TP 0.007; wash 0.035. (b) Effects of thiopentone upon ATP-K^+ channels. Outside-out patch, holding potential + 50 mV, symmetrical 140 mM KCl. Bath (extracellular) solution C, pipette (intracellular) solution D. Application of thiopentone (300 μM) to the bath resulted in a substantial and reversible decrease in single channel activity. The values of $N_f \cdot P_o$ are: control 1.490; TP 0.035; wash 1.538. (c) Lack of action of phenobarbitone on ATP-K^+ channel currents. Outside out membrane patch, under the conditions described in (b). Phenobarbitone (300 μM) applied to the bath produced no inhibition of ATP-K^+ channel activity. The values of $N_f \cdot P_o$ are: control 1.994; phenobarbitone 1.730; wash 1.595. (d) Alloxan-induced inhibition of ATP-K^+ channel currents. Outside-out patch, holding potential +50 mV, symmetrical 140 mM KCl. Bath (extracellular) solution C, pipette (intracellular) solution E. Alloxan (1 mM) produced an inhibition of ATP-K^+ channel activity that was poorly reversed on wash. The values of $N_f \cdot P_o$ are: control 3.684; alloxan 1.201; wash 1.505.

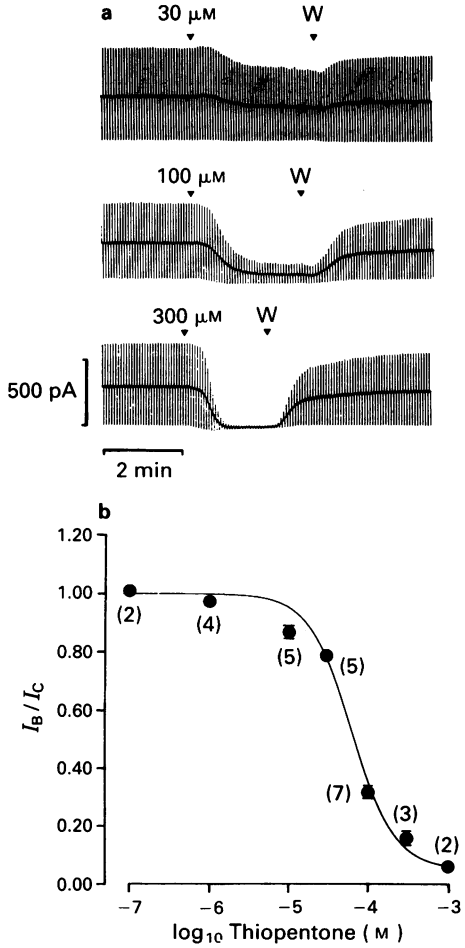

Figure 2 Effects of thiopentone upon whole-cell ATP-K^+ currents. (a) Whole-cell ATP-K^+ currents were recorded from cells bathed in normal physiological saline (solution A), with solution D in the recording pipette. The cell membrane was clamped at −70 mV and alternate ±10 mV voltage pulses, 200 ms in duration, were applied at 2 s intervals. The current responses are denoted by the vertical lines. Thiopentone induced a concentration-dependent inhibition of whole-cell ATP-K^+ currents, indicated by a decrease in the size of the current responses and a concomitant shift in the holding current (indicated by the horizontal line). The effects of thiopentone are readily reversed upon removal of the drug (W). (b) Concentration-response relationship for the inhibition of whole-cell ATP-K^+ currents by thiopentone. This is plotted as the ratio of the current in the presence of the barbiturate (I_B) to that preceding application of the drug (I_C). Data to construct the graph were obtained from a total of 10 cells, with the number of individual determinations shown in parentheses adjacent to the corresponding data point. Vertical lines indicate the s.e.mean. The line shown was fitted to the Hill equation and has an IC_{50} of 62 μM.

upon removal of the drug (Figure 2a) and it was noticeable that the on-rate was faster than the off-rate (although no quantitative assessment has been made). A plot of the ratio (I_B/I_C) of current amplitude during drug exposure (I_B) to that immediately preceding drug administration (I_C) against the logarithm of the concentration of thiopentone, using data obtained from a total of 10 cells shows a sigmoidal concentration-response relationship with an IC_{50} of 62 μM (Figure 2b). Pentobarbitone and secobarbitone, like thiopentone, reversibly inhibited whole-cell ATP-K^+ currents in a concentration-dependent manner but were much less effective than thiopentone. This is reflected in their concentration-response curves, which result in IC_{50} values of 250 μM (9 cells) and 360 μM (7 cells) respectively indicating a four and six fold lower potency than thiopentone. In agreement with the single channel studies, phenobarbitone proved to be a weak inhibitor of whole-cell ATP-K^+ currents. Application of 1 to 100 μM phenobarbitone had little effect, whilst 1 mM resulted in an

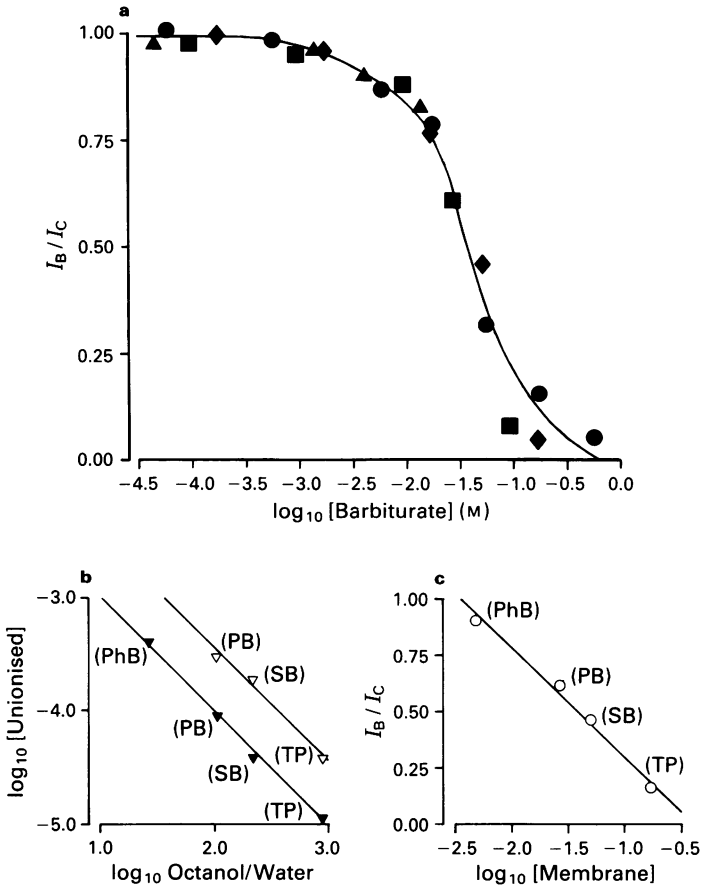

Figure 3 (a) Plot of I_B/I_C versus the $\log_{10}$ of the apparent membrane concentration for each barbiturate. The concentration-response relationship for all 4 barbiturates is fitted by a single sigmoidal curve (fitted by eye). The symbols represent (●) thiopentone, (◆) secobarbitone, (■) pentobarbitone and (▲) phenobarbitone. (b) Plot of the $\log_{10}$ concentration of unionized barbiturate required to produce 15% (▼) and 50% (▽) inhibition of whole-cell ATP-K$^+$ currents, against the $\log_{10}$ octanol/water partition coefficient ($\log_{10}$ P). Each point is identified as follows: thiopentone (TP) secobarbitone (SB), pentobarbitone (PB) and phenobarbitone (PhB). Both lines, fitted by a least squares fitting routine, have correlation coefficients of $r = 0.99$ and slopes of 1.0. (c) Plot of I_B/I_C at an aqueous concentration of 300 μM for the four barbiturates examined, against the $\log_{10}$ of their apparent membrane concentration. The abbreviations are the same as in (b). The line, fitted by a least squares fitting routine, has a correlation coefficient of $r = 0.99$.

approximate 15% inhibition of the ATP-K$^+$ currents (10 cells). Furthermore, the presence of 0.1 mM Mg-ATP in the whole-cell dialysing solution (solution F) did not alter the inhibitory effect of secobarbitone (0.1–300 μM) on the ATP-K$^+$ current ($n = 3$) indicating that binding of ATP to its inhibitory site does not modify the effectiveness of the barbiturates.

Lipid solubility and ATP-K$^+$ channel inhibition

One might surmise from the data described above that the four barbiturates tested exhibit a wide range of potencies with regard to ATP-K$^+$ channel inhibition. In order to establish whether both 'high' (e.g. thiopentone) and 'low' (e.g. phenobarbitone) potency barbiturates were likely to achieve similar concentrations in the membrane, their 'apparent' membrane concentrations were calculated, taking into account their degree of ionization and lipid solubility (in octanol). When the apparent membrane concentration of each barbiturate was calculated for a given degree of channel inhibition, the values obtained were almost identical and a plot of I_B/I_C against the apparent membrane concentration for all four barbiturates show that the data points closely follow a single sigmoidal relationship (Figure 3a) with an IC_{50} for their apparent membrane concentration of 40 mM. Furthermore there is an excellent correlation between the aqueous concentration of unionized barbiturate required to achieve a given degree of inhibition and its octanol/water partition coefficient (Figure 3b), indicating that the barbiturate potency is related to its solubility in octanol and therefore presumably membrane lipid. This is more clearly seen in a plot of the inhibition induced by 300 μM of each barbiturate against the equivalent $\log_{10}$ apparent membrane concentration (Figure 3c) which shows that the degree of inhibition is proportional to the amount of barbiturate in the membrane. Hence from these data it is likely that diffusion into the plasma membrane is an important step for barbiturate-induced ATP-K$^+$ channel inhibition.

In order to examine further the interaction of barbiturates with the ATP-K$^+$ channel the effect of concurrent application of thiopentone and phenobarbitone on whole-cell ATP-K$^+$ currents was examined. Phenobarbitone alone, at a concentration of 300 μM, induced a 10 ± 2% ($n = 4$) inhibition of the ATP-K$^+$ current, and this was increased to 31 ± 7% ($n = 4$) in the presence of 30 μM thiopentone (Figure 4a). The degree of ATP-K$^+$ current inhibition elicited by 30 μM thiopentone alone, in the absence of phenobarbitone, was 21 ± 1% ($n = 5$), a value similar to the contribution made by this barbiturate in the presence of phenobarbitone (300 μM), suggesting the effect of the two compounds was additive. The total apparent membrane concentration of the two barbiturates at these concentrations was approximately 21 mM and from the curve shown in Figure 3a this concentration correlates with a ~30% inhibition of ATP-K$^+$ activity ($I_B/I_C = 0.7$) which is identical to that observed experimentally.

The excellent correlation between the effectiveness of the barbiturates and lipid solubility indicates that their inhibitory effect upon ATP-K$^+$ channel activity is mediated by the uncharged form of the molecules. This was further substantiated by examining the effectiveness of thiopentone (pKa = 7.6) at pH 9, where it is 96.2% ionised, in comparison

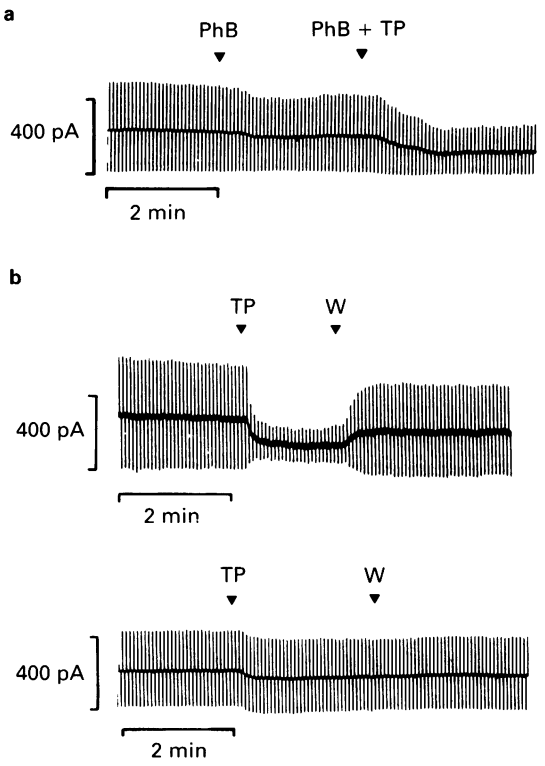

Figure 4 (a) Effect of concurrent application of phenobarbitone (PhB) and thiopentone (TP) on whole-cell ATP-K^+ currents. Application of phenobarbitone (300 μM) induced a slight inhibition of the whole-cell ATP-K^+ current, which was further decreased upon concomitant application of thiopentone (30 μM). (b) Effect of thiopentone (TP) upon whole-cell ATP-K^+ currents at different values of pH. The cells were bathed in a physiological saline which was buffered with Tris at pH 7.4 or 9.0 (solution G). Thiopentone (300 μM) at pH 7.4 (upper trace) induced a substantial decrease in the ATP-K^+ current, which was reversible upon washing (W), whereas at pH 9 (lower trace), only a weak inhibition of the ATP-K^+ currents occurred. In both (a) and (b), the cell membrane was clamped at -70 mV and alternate ± 10 mV voltage pulses, 200 ms in duration, were applied at 2 s intervals. The current responses are denoted by the vertical lines. The cells were maintained in solution A with solution D in the recording pipette.

to pH 7.4 where it is only 39% ionised. In these experiments cells were bathed in a normal physiological saline containing 10 mM Tris as buffer (solution G). The inhibitory effect of thiopentone (100 μM) at pH 7.4 in the presence of Tris buffer was $62 \pm 4\%$ ($n = 4$) and was not significantly different from the $68 \pm 5\%$ ($n = 7$) inhibition observed in the presence of HEPES buffer at pH 7.4. In contrast, application of thiopentone (100 μM) at pH 9 reduced the whole-cell ATP-K^+ current by $15 \pm 5\%$ ($n = 6$), with little reversal occurring upon washout of the drug (Figure 4b). However, on consideration of the apparent membrane concentration of thiopentone under these conditions (3.65 mM; $\log_{10} = -2.46$) this effect was slightly greater than that expected from the graph shown in Figure 3a, which indicates that the ionised species may be responsible for some of the blockade.

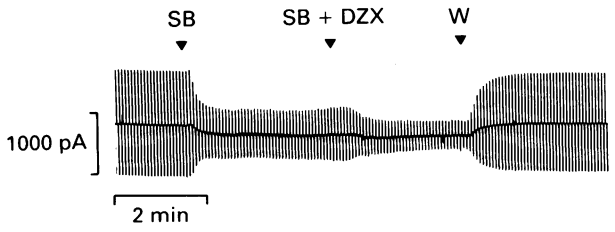

Figure 5 Lack of effect 0.6 mM diazoxide (DZX) upon ATP-K^+ channel current inhibition induced by 300 μM secobarbitone (SB). The cell was maintained in solution A with solution D in the recording pipette. The combined effect of the two drugs was readily reversed upon wash (W). The cell membrane was clamped at -70 mV and alternate ± 10 mV voltage pulses, 200 ms in duration, were applied at 2 s intervals. The current responses are denoted by the vertical lines.

Effect of diazoxide on barbiturate-induced ATP-K^+ channel inhibition

The inhibition of ATP-K^+ currents by the barbiturates is similar to that of sulphonylureas like tolbutamide (Sturgess *et al.*, 1988) which are specific inhibitors of this channel and are thought to approach their site of action via membrane lipid (Zunkler *et al.*, 1989). One way of determining whether barbiturates share a common mechanism or site of action with the sulphonylureas was to examine whether diazoxide reversed barbiturate-induced ATP-K^+ channel inhibition in the same manner as it does tolbutamide-induced channel inhibition (Sturgess *et al.*, 1988). Inhibition of whole-cell ATP-K^+ currents induced by either 100 or 300 μM secobarbitone, was not reversed by diazoxide at concentrations of 0.3 or 0.6 mM ($n = 4$ cells), which under conditions of low internal Mg^{2+} and ATP concentrations (Kozlowski *et al.*, 1989b), elicited a further inhibitory effect (Figure 5).

Specificity of thiopentone for ATP-K^+ channels

In order to determine whether the barbiturates, like the sulphonylureas, exhibit specificity of action for ATP-K^+ channels, the effects of thiopentone at a concentration of 100 μM were examined on voltage-activated currents in CRI-G1 cells.

Under conditions where both sodium and calcium entry were blocked (Kozlowski *et al.*, 1991) thiopentone reduced the magnitude of the delayed outward K^+ current, an effect which was reversible upon washout of the drug (Figure 6a). This

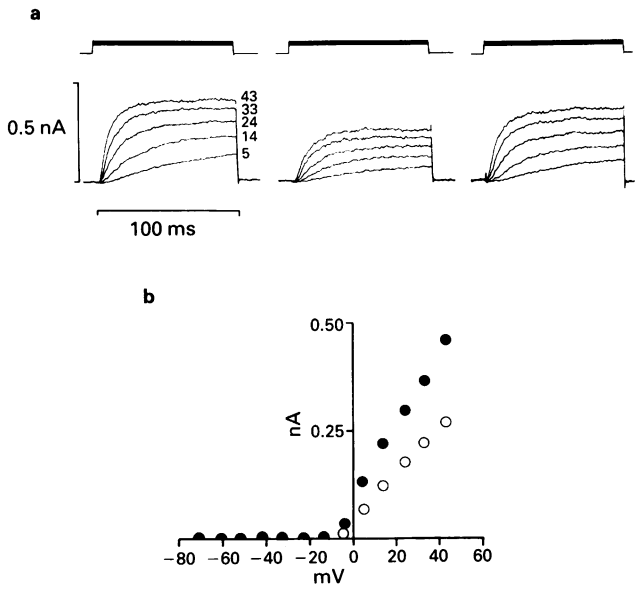

Figure 6 Effect of thiopentone upon delayed outward K^+ currents. (a) Data were obtained from a cell bathed in normal physiological saline (solution A) to which $CdCl_2$ (1 mM) and tetrodotoxin (TTX) (300 nM) had been added to block inward Ca^{2+} and Na^+ currents, respectively. The recording pipette contained solution H. Currents were elicited by 100 ms pulses applied at a frequency of 1 Hz from a holding potential of -80 mV. Each current record represents the average of three leak subtracted K^+ currents elicited at the test potentials shown on the control trace (left panel). Application of 100 μM thiopentone (centre panel) resulted in a reduction in the peak current amplitude which was partially reversible upon removal of the barbiturate (right panel). (b) Current-voltage relationship for K^+ currents in the absence (●) and presence (○) of thiopentone (100 μM) which incorporates the data shown in (a). Each point represents the average of three currents.

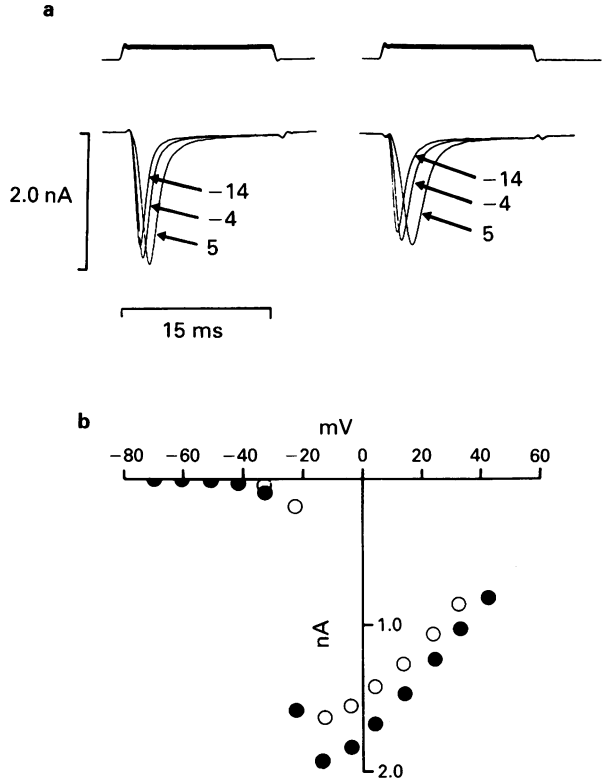

Figure 7 Effect of thiopentone upon voltage-activated inward Na^+ currents. (a) Data were obtained from a cell bathed in normal physiological saline (solution A) to which $CdCl_2$ (1 mM), tetraethylammonium (20 mM) and 4-aminopyridine (4 mM) had been added to block inward Ca^{2+} currents and any outward currents through K^+ channels. The recording pipette contained solution I. Currents were elicited by 15 ms pulses applied at a frequency of 1 Hz from a holding potential of −80 mV. Each current record represents the average of four leak subtracted Na^+ currents elicited at the test potentials indicated on the figure. In the presence of 100 μM thiopentone (right panel) there was a slight reduction in the peak current amplitude and a slight decrease in the rate of activation and inactivation when compared to control levels (left panel). (b) Current-voltage relationship for Na^+ currents in the absence (●) and presence (○) of thiopentone (100 μM) which incorporates the data shown in (a). Each point represents the average of four currents.

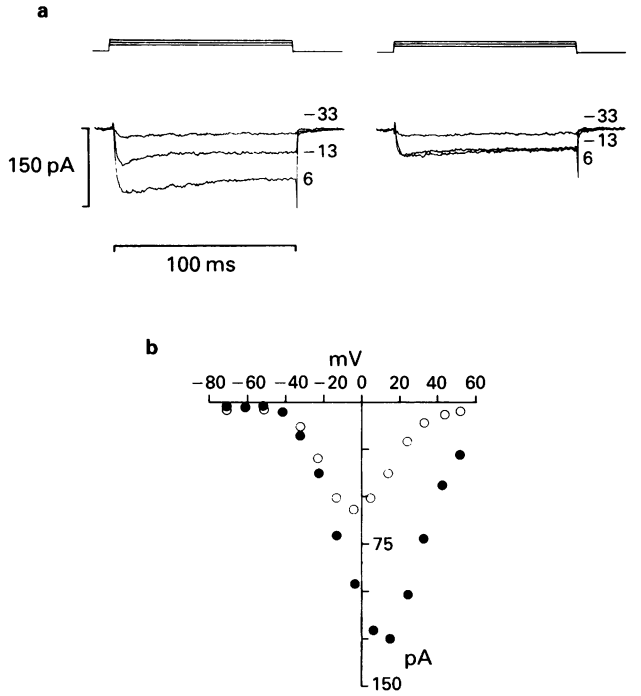

Figure 8 Effect of thiopentone upon voltage-activated inward Ca^{2+} currents. (a) Data were obtained from a cell bathed in a physiological saline (solution A) to which tetrodotoxin (300 nM) had been added, and the concentration of $CaCl_2$ raised to 10 mM to enhance the amplitude of the inward current. The recording pipette contained solution J. Currents were elicited by 100 ms pulses applied at a frequency of 1 Hz from a holding potential of −80 mV. Each current record represents the average of three leak subtracted Ca^{2+} currents elicted at the test potentials indicated on the figure. Application of 100 μM thiopentone (right panel) to the cell reduced the amplitude of the peak inward current with respect to control levels (left panel). (b) Current-voltage relationship for peak Ca^{2+} currents in the absence (●) and presence (○) of thiopentone (100 μM) which incorporates the data shown in (a). Each point represents the average of three currents, as described above.

inhibitory action was observed as a reduction of the slope for the current-voltage relationship of the peak (plateau) outward current (Figure 6b) with little effect on the time-to-peak. In the absence of thiopentone the average slope for the current-voltage relationship was 7.53 ± 0.98 nS (n = 8), whilst in the presence of the drug it was 5.76 ± 0.97 nS (n = 8). Thus, thiopentone at a concentration of 100 μM decreased the magnitude of the outward K^+ current by 25.9 ± 5.4% (n = 8). However, its activation threshold remained unaffected, as outward K^+ currents were detected at test potentials of −16.2 ± 2.2 mV (n = 8) and −14.9 ± 2.0 mV (n = 8) in the absence and presence of 100 μM thiopentone, respectively.

The amplitude of voltage-activated inward Na^+ currents was only slightly reduced by this concentration of thiopentone (Figure 7a), an effect which was poorly reversed upon washout of the drug (data not shown). This inhibitory action is illustrated in Figure 7b which is a current-voltage relationship incorporating the data shown in Figure 7a. Thiopentone reduced the magnitude of the peak inward Na^+ current (which ranged from −0.29 to −1.92 nA and −0.27 to −1.61 nA in the absence and presence of the drug, respectively) by only 8.0 ± 5.25% (n = 6). Furthermore, no change was observed in the test potential at which the peak inward Na^+ current was evoked being +2.3 ± 4.8 mV (n = 6) in the presence, and +2.9 ± 5.2 mV (n = 6) in the absence of thiopentone. However, thiopentone did seem to alter the kinetics of the Na^+ current, since there appeared to be a slowing of both the activation and inactivation rate for the currents (Figure 7a) but this effect was not examined in detail. Voltage-dependent Ca^{2+} currents were much more sensitive to thiopentone than Na^+ or K^+ currents. Thiopentone (100 μM) reduced the peak Ca^{2+} current (which ranged from −93 to −125 pA and −46 to −72 pA in the absence and presence of the drug, respectively) by 43.8 ± 10.6% (n = 3) without any noticeable change in current time course. Thiopentone (100 μM) also induced a shift in the potential at which the peak calcium current was elicited. An example of these effects is illustrated in Figure 8a with the corresponding current-voltage relationship shown in Figure 8b.

Discussion

It is clear from this study that thiopentone inhibits a variety of ionic currents in the insulin-secreting cell-line CRI-G1, and that ATP-K^+ channel activity is the most susceptible. The resting membrane potential of pancreatic β-cells (and insulin-secreting cell-lines) is largely determined by the activity of ATP-K^+ channels particularly in the absence or in the presence of low concentrations of extracellular glucose (Ashcroft *et al.*, 1988). A reduction of ATP-K^+ channel activity under these conditions will result in β-cell depolarization, calcium entry and insulin release (Ashford, 1990). Thus inhibition of the activity of this channel elicited by thiopentone in cell-attached recordings from cells exposed to nutrient-free solution (where ATP-K^+ channels are very active) would explain the secretagogue action reported for this drug in β-cells at low

extracellular glucose concentrations (Gonçalves *et al.*, 1986). Consequently, thiopentone, (and probably pentobarbitone and secobarbitone, which were also shown to be effective inhibitors of ATP-K^+ channel currents) can act as initiators of insulin secretion and so mimic the action of the sulphonylurea group of drugs which are considered to be specific inhibitors of the ATP-K^+ channel (for a review, see Ashford, 1990).

However in the majority of *in vivo* and *in vitro* studies, barbiturates such as pentobarbitone decrease insulin levels in whole animals (Aynsley-Green *et al.*, 1973; Renauld & Sverdlik, 1975) or insulin release from β-cells (Panten *et al.*, 1973; Hellman, 1977). This inhibition is observed in β-cells which have been stimulated with glucose (i.e. the ATP-K^+ channels are mostly closed) and are consequently firing action potentials. One plausible explanation for this action is that pentobarbitone (and presumably other barbiturates) inhibit the voltage-dependent calcium current which underlies the depolarization phase of the action potential (Ashcroft & Rorsman, 1990). Our results certainly support this possibility as 100 μM thiopentone, a concentration often attained in the blood plasma during thiopentone anaesthesia (Heinemayer, 1987) induces a $\sim$44% block of this current in CRI-G1 insulin-secreting cells.

Hence we propose that the variable reports in the literature of barbiturate actions on blood insulin or glucose levels are as a result of their inhibition if these two types of channel current in combination with variable levels of extracellular glucose (i.e. whether the cells are stimulated or not). For example, at low (fasting) glucose levels, two opposing influences would be apparent, a secretagogue action through β-cell depolarization (due to inhibition of ATP-K^+ channel current) and an anti-secretory action (reduction of calcium entry). Whereas at high concentrations of extracellular glucose (stimulated cells) only an inhibitory action would be observed (due to calcium current inhibition). This contrasts with the effects of the sulphonylureas which have been reported only to inhibit ATP-K^+ channel currents in pancreatic β-cells and therefore only act as secretagogues.

Alloxan, a compound structurally related to the barbiturates was also shown to inhibit ATP-K^+ channel currents in outside-out membrane patches. This agrees with the findings of Dean & Matthews (1972), who have shown that this compound induces a rapid depolarization of the β-cell and suggested that it is mediated through an interaction with a specific membrane component. In addition, Mennear *et al.* (1976) proposed that barbituric acid, tolbutamide and alloxan compete for the same site on the β-cell membrane. In view of the findings presented here, this may be the ATP-K^+ channel. However it is not clear whether this acute effect of alloxan has any relation to its chronic diabetogenic action (severe hyperglycemia) via β-cell destruction (Rerup, 1970).

The ATP-K^+ channel block elicited by all four barbiturates and the structurally-related, diabetogenic compound alloxan, showed no voltage-dependence suggesting that the blocking action was not associated with the channel pore itself (Hille, 1984). Pentobarbitone and secobarbitone applied to the extracellular aspect of membrane patches inhibited the channel in a manner similar to thiopentone, but at a reduced potency. Phenobarbitone was found to be ineffective when applied to either the intra- or extracellular membrane surface, an effect shown to be due to its low lipid solubility. The whole-cell ATP-K^+ current data also show that the potency of the barbiturates is markedly different. However, calculation of their apparent membrane concentrations using partition coefficients derived in an octanol/water system revealed that (a) the same concentration of each barbiturate is present in the membrane for a given degree of ATP-K^+ channel inhibition and (b) a good correlation exists between the degree of channel inhibition induced by the barbiturates and their solubility in octanol. This clearly indicates that hydrophobicity and lipophilicity are important in the inhibitory effects of these compounds upon ATP-K^+ channels. This hypothesis is substantiated by the observation that the effects of phenobarbitone and thiopentone are additive, and that the degree of inhibition is related directly to the apparent membrane concentration of the two barbiturates. Furthermore, thiopentone applied at pH 9, where it is largely ionised, induced only a small degree of inhibition, an action which reflects the decreased apparent membrane concentration of the drug. Therefore it is likely that the unionised form is the active inhibitory species of the molecule, in agreement with the findings of Blaustein (1968), Narahashi *et al.* (1971) and Zbicz & Wilson (1981) on voltage-activated conductances.

As the membrane concentration of the barbiturates is clearly important for determining the degree of ATP-K^+ channel inhibition, this may imply that, as suggested for general anaesthetics, they act by a non-specific membrane interaction such as an alteration of the physical characteristics of the membrane lipid, thereby inducing changes in membrane protein function (e.g. Novak & Swift, 1972; Lee, 1976; Harris & Schroeder, 1980) or by an action on specific hydrophobic protein regions inducing an allosteric change in channel gating (Richards *et al.*, 1978). Alternatively, the barbiturates may interact with the ATP-binding site of the channel directly as they are adenosine (A_1) receptor antagonists (Lohse *et al.*, 1985) and an adenosine receptive part of the ATP recognition site exists (Weik & Neumcke, 1989). It is also possible that the barbiturates interact with the sulphonylurea receptor although, unlike the ATP-K^+ channel inhibition induced by tolbutamide (Trube *et al.*, 1986; Dunne *et al.*, 1987; Sturgess *et al.*, 1988) the inhibition induced by secobarbitone is not reversed by diazoxide.

In view of the diverse actions of barbiturates on excitable cells it was pertinent to determine the specificity of ATP-K^+ channel inhibition in comparison to voltage-activated currents. The results indicate that thiopentone (100 μM) also reduces the magnitude of voltage-activated Ca^{2+}, Na^+ and K^+ currents in CRI-G1 cells. Assuming the potency of thiopentone on the ATP-K^+ channel currents is 1.00, its relative potency for Na^+, K^+ and Ca^{2+} currents is 0.12, 0.38 and 0.64 respectively, thereby indicating some degree of selectivity for ATP-K^+ channels over the voltage-dependent ionic conductances. There have been several reports that barbiturates affect Ca^{2+} influx, causing a reduction in the duration of Ca^{2+}-dependent action potentials (Heyer & MacDonald, 1982), and of synaptosomal Ca^{2+} influx (Blaustein & Ector, 1985). More recently Gross & Macdonald (1988), using mouse neurones isolated from dorsal root ganglia have reported that pentobarbitone (500 μM) and phenobarbitone (2 mM) selectively reduce the amplitude of the L-type Ca^{2+} current, with no effect on T- or N-type currents. This would agree with the findings presented in this study as peak inward Ca^{2+} current is probably carried largely by L-type channels in insulin-secreting cells (Smith *et al.*, 1989) and this cell-line (Kozlowski *et al.*, 1991).

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The neuroprotective action of dizocilpine (MK-801) in the rat middle cerebral artery occlusion model of focal ischaemia

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1 An acute model of focal ischaemia, which involves permanent occlusion of the middle cerebral artery of the rat with 4 h survival, was used to find the minimum effective plasma concentration of dizocilpine (MK-801) and to determine its dose-effect relationship.

2 MK-801 was administered at the time of occlusion and was given as an i.v. bolus followed by an infusion for 4 h to maintain a steady state plasma concentration of the drug throughout the study. MK-801 was given at 3 dose levels; 0.04 mg kg^{-1} i.v. bolus + 0.6 μg kg^{-1} min^{-1} infusion; 0.12 mg kg^{-1} i.v. bolus + 1.8 μg kg^{-1} min^{-1} infusion; 0.4 mg kg^{-1} i.v. bolus + 6 μg kg^{-1} min^{-1} infusion, which gave mean plasma levels over the 4 h of 8.0 ng ml^{-1}, 18.9 ng ml^{-1} and 113.2 ng ml^{-1} respectively.

3 MK-801 at 8.0 ng ml^{-1} gave 10% reduction in the volume of ischaemic brain damage in the cerebral cortex which just reached significance. The middle dose of MK-801 (18.9 ng ml^{-1}) gave a highly significant reduction in the volume of ischaemic brain damage in the cerebral cortex and hemisphere, volumes of ischaemic tissue being reduced by 60% and 50% compared to saline-treated animals, respectively. The highest plasma concentration of MK-801 (113.2 ng ml^{-1}) resulted in a 35% reduction in the volume of hemispheric damage and a 40% reduction in the volume of cortical damage, which were significant.

4 The reduction in the amount of protection afforded by the highest dose of MK-801 may be due to the hypotensive effect of this dose. There was no protection against the volume of damage in the caudate nucleus for any of the doses of MK-801 tested.

5 Therefore the minimum effective plasma concentration of MK-801 was 8.0 ng ml^{-1}, although the greatest protection was seen with a plasma level of 18.9 ng ml^{-1}. This correlates well with the concentration of MK-801 required to block N-methyl-D-aspartate (NMDA) receptors and prevent NMDA receptor mediated neurotoxicity *in vitro*.

Keywords: Excitatory amino acids; glutamate; cerebrovascular disease; neurotoxicity; MK-801; NMDA antagonists; ischaemia; neuroprotection; focal ischaemia

Introduction

The neuronal degeneration which results from a period of cerebral ischaemia is thought to be due, at least in part, to an overexcitation at synapses using L-glutamate or L-aspartate as their neurotransmitter (Rothman & Olney, 1986; Choi, 1988). The initial evidence for this came mainly from transient forebrain ischaemia studies in which a massive increase in the extracellular concentrations of glutamate and aspartate was seen following ischaemia (Benveniste *et al.*, 1984; Hagberg *et al.*, 1985), and lesions of glutamatergic pathways projecting to the hippocampus ameliorated neuronal damage to this region (Wieloch *et al.*, 1985; Benveniste *et al.*, 1989).

The N-methyl-D-aspartate (NMDA) subtype of excitatory amino acid receptors appears to be the key receptor involved in ischaemia-induced neuronal degeneration for a number of reasons. Firstly, an important property of the NMDA receptor is that its associated ion channel is highly permeable to Ca^{2+} (MacDermott *et al.*, 1986). Thus, under ischaemic conditions, NMDA receptor activation, due to the excessive glutamate release, results in large increases in intracellular [Ca^{2+}], which are thought to be involved in pathways resulting in cell death (Siesjö, 1981). These increases in intracellular [Ca^{2+}] are mirrored by a large decrease in extracellular Ca^{2+} levels during the ischaemic episode (Benveniste *et al.*, 1988; Andiné *et al.*, 1988). Secondly, the neurones which are selectively vulnerable to brief periods of ischaemia (Pulsinelli & Brierley, 1979; Kirino, 1982) possess the highest density of NMDA receptors in the brain (Monaghan & Cotman, 1985). Finally, studies with selective NMDA antagonists (Simon *et al.*, 1984; Gill *et al.*, 1987; 1988; Boast *et al.*, 1988) have shown that these are neuroprotective in animal models of transient forebrain ischaemia, although there have been some negative results reported as well (Block & Pullsinelli, 1987; Jensen & Auer, 1988; Wieloch *et al.*, 1989).

MK-801 is one of the most potent and selective NMDA antagonists available (Wong *et al.*, 1986). We have previously demonstrated it to be neuroprotective in a gerbil model of transient forebrain ischaemia when given prior to or up to 24 h post-ischaemia. The neuronal damage seen in the gerbil is of the delayed type. That is, neuronal damage does not occur immediately but occurs over a 24–48 h period (Kirino, 1982; Gill *et al.*, 1987). This phenomenon is also seen in human post mortem tissue from patients who have suffered a cardiac arrest (Petito *et al.*, 1987).

Following this work in transient forebrain ischaemia studies, NMDA antagonists have subsequently been shown to be potent neuroprotective agents in animal models of focal cerebral ischaemia (Duverger *et al.*, 1987; Ozyurt *et al.*, 1988; Park *et al.*, 1988a,b; Bielenburg, 1989) which are more akin to stroke in man. The protective effects of NMDA antagonists in these models appear to be more reproducible than in transient forebrain ischaemia models. MK-801, as a single dose, was found to be neuroprotective against cortical infarction when given prior to or post-ischaemia following permanent middle cerebral artery (MCA) occlusion in the rat or cat (Ozyurt *et al.*, 1988; Park *et al.*, 1988a,b). The aim of the present series of experiments was to determine the minimum effective plasma concentration of MK-801 in a focal ischaemia model in the rat and to investigate its dose-effect relationship. Therefore, we used an infusion regimen to maintain a steady state plasma level of MK-801 throughout the ischaemic period. For these experiments we used the acute rat MCA occlusion model of Tamura *et al.* (1981) because it enabled continuous monitor-

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ing of all physiological parameters, and because it allowed us to monitor closely the plasma levels of MK-801 throughout the study.

Methods

Male Sprague Dawley rats weighing 300–350 g were used for these studies; the animals were maintained on a 12 h light:dark cycle and fed *ad libitum* prior to experimentation.

The method used for permanent MCA occlusion was essentially as described by Tamura *et al.* (1981) and Shigeno *et al.* (1985). Briefly rats were anaesthetized with a mixture of 4% isoflurane, 30% oxygen and 70% nitrous oxide, a trachaeostomy was performed and the animals ventilated with a mixture of 2–3% isoflurane, 30% oxygen and 70% nitrous oxide.

The left femoral artery and vein were cannulated to enable continuous blood pressure recordings, blood sampling, injections and infusions. All animals then underwent a subtemporal craniectomy and exposure of the left MCA (Tamura *et al.*, 1981), the zygomatic arch was left intact. The stem of the artery and its lenticulostriate branches were occluded with microbipolar coagulation. Ventilation was subsequently maintained with a mixture of 1% isoflurane, 30% oxygen and 70% nitrous oxide. The blood gases were monitored prior to MCA occlusion then every hour following the occlusion for the duration of the experiment. They were maintained at $Paco_2$ of >30 mmHg and Pao_2 of >100 mmHg, the animals were also kept normothermic at 37 ± 0.5°C, throughout the experiment, by use of a rectal probe and heating blanket (Harvard apparatus, U.K.). The blood pressure was continuously monitored during the experiment; it was maintained at 80 mmHg for the surgical procedures and following MCA occlusion it was allowed to return to normal.

The animals were maintained under these conditions for 4 h after which they were perfusion fixed with 40% formaldehyde, glacial acetic acid and methanol (FAM; 1:1:8, v/v/v). The brains were processed and embedded in paraffin wax, 8 μm sections were stained with haematoxylin-eosin or with a combination of cresyl violet and luxol fast blue. The sections were examined by light microscopy and areas of early infarction were delineated at 8 preselected coronal levels from anterior 10.5 mm to anterior 1.0 mm (Konig & Klippel, 1963). This was done blind to the drug treatment of the animals. The areas of brain damage were drawn on scale diagrams ($\times 4$ actual size) of forebrain and measured in terms of hemisphere, cortex and caudate on an image analyzer (Quantimet 760, Cambridge Instruments). The areas of ischaemic damage were used to determine the total volume of ischaemic tissue in each brain (Osborne *et al.*, 1987), which was done by integration of areas with the distance between each level. The end points for integration for the cortex and hemisphere were anterior 12.5 and posterior 0.05. The amount of ischaemic damage was expressed in absolute terms (mm^3) for hemisphere, cortex and caudate.

MK-801 was tested at 3 doses, 0.04 mg kg^{-1} as an i.v. bolus + 0.6 μg kg^{-1} min^{-1} infusion; 0.12 mg kg^{-1} i.v. bolus + 1.8 μg kg^{-1} min^{-1} infusion and 0.4 mg kg^{-1} i.v. bolus + 6 μg kg^{-1} min^{-1} infusion. Initial dosing was started immediately following MCA occlusion, the bolus dose was administered over a 30 s period and the infusion was continued for the duration of the experiment (4 h). Plasma samples (100 μl) were taken prior to MCA occlusion and then every hour following occlusion. The plasma samples were assayed in the MK-801 radioimmunoassay described below. The control groups of animals received 1 ml kg^{-1} i.v. bolus of saline followed by an infusion of saline at a rate of 0.0298 ml min^{-1} for 4 h. There were 10–12 animals in each of the groups.

MK-801 was measured in plasma samples by radioimmunoassay (RIA) (Hichens *et al.*, 1991). Briefly, antisera were raised to N-glutaryl-MK-801 coupled to bovine serum albumin, and the radioligand was an iodotyramine conjugate of the same derivative. Specificity was for the N-acyl derivatives and the drug was extracted from plasma and N-acetylated prior to using a double antibody RIA procedure. Metabolites were eliminated by the extraction method. Recovery (51 ± 3.8%) was independent of concentration. Inter- and intra-assay coefficients of variation were 12% and 3.8%, respectively.

Statistical analysis

Differences between the volume of ischaemic damage in the control and MK-801-treated animals were tested by analysis of variance (ANOVA) with a Bonferroni correction; this was done for each of the brain regions (hemisphere, cortex and caudate). The curves for the area of ischaemic damage in the cortex were analysed by BMDP 2v (BMDP Statistical Software, Ireland), which is an analysis of variance and covariance test with repeated measures. The area of ischaemic damage at each coronal plane was compared between control and MK-801-treated animals by the BMDP 2v analysis of variance test with repeated measures. All data are presented as the mean $\pm$ s.e.mean for n animals.

Results

The lowest dose of MK-801 (0.04 mg kg^{-1} i.v. bolus + 0.6 μg kg^{-1} min^{-1}) gave a plasma concentration in the range of 8–12 ng ml^{-1} with a mean plasma concentration of 8.0 ng ml^{-1} over the 4 h period (Figure 1*a*). MK-801 at a dose of 0.12 mg kg^{-1} i.v. bolus + 1.8 μg kg^{-1} min^{-1} as an infusion gave a range of 10–52 ng ml^{-1} with a mean plasma concentration of 18.9 ng ml^{-1} over the 4 h period (Figure 1b). The highest dose of MK-801 of 0.4 mg kg^{-1} i.v. bolus + 6 μg kg^{-1} min^{-1} gave plasma levels in the range of 100–140 ng ml^{-1} with a mean of 113.2 ng ml^{-1} over the 4 h period (Figure 1c). The early peak plasma concentration of MK-801 was not monitored in this study, however, a study using the same dosing regime as this (Willis *et al.*, 1991) has shown that the initial peak levels of MK-801 in both plasma and CSF which occur following the bolus dose reach equilibrium within 10 min. Therefore with these dosing regimes a steady state plasma level of MK-801 was achieved during the 4 h period of ischaemia.

There were no significant differences observed for any of the MK-801-treated groups and their saline-treated controls with respect to respiratory blood gas levels or blood glucose levels (Table 1). The rectal temperature of all animals was maintained at 37 ± 0.5°C throughout the experimental period using a thermostatically controlled blanket. Ischaemic damage was observed only within the territory of the occluded MCA, that is, in the dorsolateral cortex and in the neostriatum. These areas showed the morphological characteristics of early ischaemic cell change with microvaculation, shrinkage and triangulation of the nucleus and cytoplasm. The nucleus also became very darkly stained and pyknotic. The histological appearance of ischaemic brain tissue in rats treated with MK-801 was similar to that in the saline-treated group in areas where ischaemic damage was present but in areas showing protection the cells appeared normal.

The lowest dosing regime of MK-801, which resulted in a mean plasma concentration of 8.0 ng ml^{-1}, gave only just significant reduction of the volume of ischaemic brain damage in the cerebral cortex (ANOVA; $F = 4.51$, $P < 0.05$), the volume of ischaemic tissue was reduced by 10% compared with saline treated controls (Figure 2a), but not in the cerebral hemisphere (ANOVA; $F = 4.13$, $P > 0.05$) or caudate nucleus (ANOVA; $F = 0.06$; $P > 0.8$; Figure 2a). The middle dose of MK-801 resulted in a mean plasma concentration of 18.9 ng ml^{-1} over the 4 h period, this gave a highly significant reduction in the volume of ischaemic brain damage in the cerebral cortex (ANOVA; $F = 111.63$, $P < 0.0001$) and hemisphere (ANOVA; $F = 115.58$, $P < 0.0001$) volumes of isch-

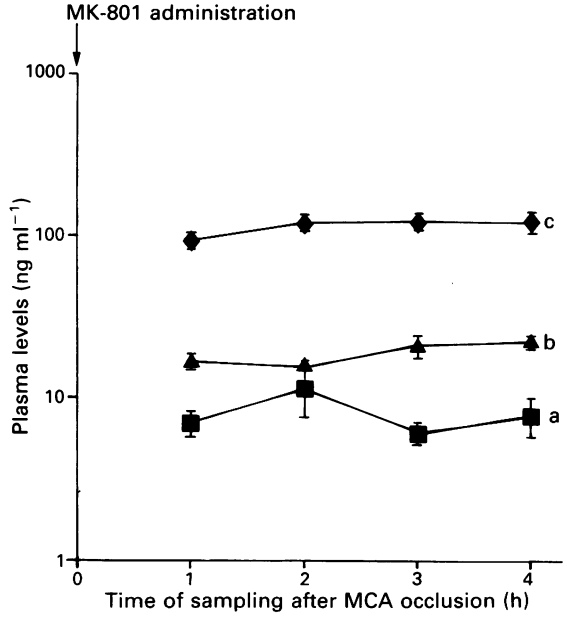

Figure 1 Plasma levels of MK-801 at different times of sampling following middle cerebral artery (MCA) occlusion. The different dosing regimes of MK-801 were all initiated immediately after MCA occlusion. (a) Plasma levels of MK-801 following a dose of $0.04\,mg\,kg^{-1}$ i.v. bolus + $0.6\,\mu g\,kg^{-1}\,min^{-1}$ infusion for 4 h. The mean plasma concentration over this period was $8.0\,ng\,ml^{-1}$. (b) The plasma concentration of MK-801 achieved by a dose of $0.12\,mg\,kg^{-1}$ i.v. bolus + $1.8\,\mu g\,kg^{-1}\,min^{-1}$ infusion for 4 h. The mean plasma concentration was $18.9\,ng\,ml^{-1}$ over the 4 h period. (c) Plasma levels of MK-801 following a dose of $0.4\,mg\,kg^{-1}$ i.v. bolus + $6\,\mu g\,kg^{-1}\,min^{-1}$ infusion for 4 h. The mean plasma level was $113.2\,ng\,ml^{-1}$. Each point on the curves represents the mean of 9–10 animals, vertical bars show s.e.mean.

aemic tissue were reduced by 60% and 50% compared to saline-treated animals, respectively (Figure 2b). There was no significant reduction in the volume of damage in the caudate nucleus (ANOVA; $F = 0.82$, $P > 0.4$) of the MK-801-treated group (Figure 2b). The highest plasma concentration of $113.2\,ng\,ml^{-1}$ was achieved with a dose of $0.4\,mg\,kg^{-1}$ i.v. bolus + $6\,\mu g\,kg^{-1}\,min^{-1}$ infusion, this resulted in a significant reduction of the volume of ischaemic brain damage in the cerebral cortex (ANOVA; $F = 8.26$, $P < 0.01$) and hemisphere (ANOVA; $F = 8.78$, $P < 0.01$); the volumes of ischaemic tissue were reduced by 40% and 35% compared with controls, respectively (Figure 2c). There was no change in the volume of caudate damage (ANOVA; $F = 1.19$, $P > 0.3$) between the control and MK-801-treated animals at this dose either.

The area of damage at different stereotactic levels was then studied for the 3 plasma concentrations of MK-801. At the plasma concentration of $8.0\,ng\,ml^{-1}$ there was no overall significant (BMDP 2v; $P = 0.053$) effect for the area of cortical damage in the control and MK-801 treated groups (Figure 3a); however, when individual stereotactic levels were compared there was a significant (BMDP 2v; $P < 0.05$) difference at 2 stereotactic levels only (Figure 3a). For the medium dose of MK-801 (mean plasma level $18.9\,ng\,ml^{-1}$) the area of ischaemic damage in the cerebral cortex was significantly (BMDP 2v; $P < 0.0001$) smaller in the MK-801 treated group than those in the saline treated group (Figure 3b). There was a significant (BMDP 2v; $P < 0.01$) reduction in the ischaemic area at each stereotactic coronal plane (Figure 3b), the greatest reductions were observed in the caudal coronal plane. However, there was no significant difference in the area of ischaemic damage for the caudate nucleus between the two groups of animals at this dose (data not shown). For the highest dose of MK-801 (mean plasma level of $113.2\,ng\,ml^{-1}$) tested, there was a significant (BMDP 2v; $P < 0.01$) decrease in the overall area of ischaemic damage in the cerebral cortex for the MK-801-treated group compared to the saline-treated controls (Figure 3c). There were also significant ($P < 0.05$) decreases in the area of damage at coronal points between anterior 7.19 and anterior 2.18 mm from bregma. There was no significant change in the area of ischaemic damage at the different stereotactic levels for the caudate nucleus ($P > 0.2$; data not shown).

The lowest plasma concentration of MK-801 of $8.0\,ng\,ml^{-1}$ produced no significant effect on the mean arterial blood pressure (MABP) of the animals (Figure 4a). However, a plasma concentration of $18.9\,ng\,ml^{-1}$ of MK-801 gave a decrease in the MABP straight after the bolus dose had been administered. The MABP was reduced significantly ($P < 0.05$) by 28 mmHg from the preinjection level although it returned to control levels within 1 h (Figure 4b). The highest dose of

Table 1 The physiological parameters for the animals measured throughout the experiment

	Pre-MCA	*1 h post*	*2 h post*	*3 h post*	*4 h post*
A					
Pao_2 Control	126 ± 7	133 ± 6	126 ± 4	129 ± 3	123 ± 3
Pao_2 MK-801	118 ± 5	122 ± 4	118 ± 4	124 ± 4	119 ± 3
$Paco_2$ Control	35 ± 1	36 ± 1	36 ± 1	34 ± 1	36 ± 1
$Paco_2$ MK-801	37 ± 1	36 ± 1	37 ± 1	35 ± 1	36 ± 1
Gluc Control	14 ± 1	8 ± 1	7 ± 1	8 ± 0	7 ± 0
Gluc MK-801	17 ± 1	9 ± 1	8 ± 0	7 ± 0	7 ± 0
B					
Pao_2 Control	141 ± 6	135 ± 7	145 ± 7	143 ± 5	136 ± 6
Pao_2 MK-801	128 ± 5	128 ± 7	144 ± 5	129 ± 4	126 ± 2
$Paco_2$ Control	35 ± 1	36 ± 1	36 ± 1	34 ± 2	37 ± 2
$Paco_2$ MK-801	38 ± 1	37 ± 1	35 ± 1	35 ± 1	35 ± 1
Gluc Control	18 ± 2	13 ± 2	12 ± 3	12 ± 2	11 ± 2
Gluc MK-801	19 ± 2	16 ± 3	14 ± 3	10 ± 2	8 ± 2
C					
$Paco_2$ Control	127 ± 6	148 ± 5	145 ± 5	144 ± 4	147 ± 5
Pao_2 MK-801	120 ± 9	135 ± 8	130 ± 4	136 ± 4	140 ± 4
$Paco_2$ Control	36 ± 2	38 ± 1	37 ± 1	36 ± 1	36 ± 1
$Paco_2$ MK-801	38 ± 2	37 ± 1	36 ± 1	36 ± 1	35 ± 1
Gluc Control	20 ± 1	11 ± 1	8 ± 1	8 ± 1	8 ± 1
Gluc MK-801	20 ± 1	8 ± 1	7 ± 1	7 ± 1	7 ± 1

The physiological parameters of animals used in these studies were measured prior to middle cerebral artery (MCA) occlusion then at 1, 2, 3 and 4 h post occlusion. The Pao_2 and $Paco_2$ values are expressed in mmHg. Plasma glucose (Gluc) concentration is given in $mmol\,l^{-1}$. (A) These are the values from the experiment in which plasma MK-801 levels were maintained at $8.0\,ng\,ml^{-1}$. (B) This group of animals had plasma MK-801 levels of $18.9\,ng\,ml^{-1}$. (C) The final group had plasma MK-801 levels of $113.2\,ng\,ml^{-1}$. Each value represents the mean ± s.e.mean from 10–12 animals.

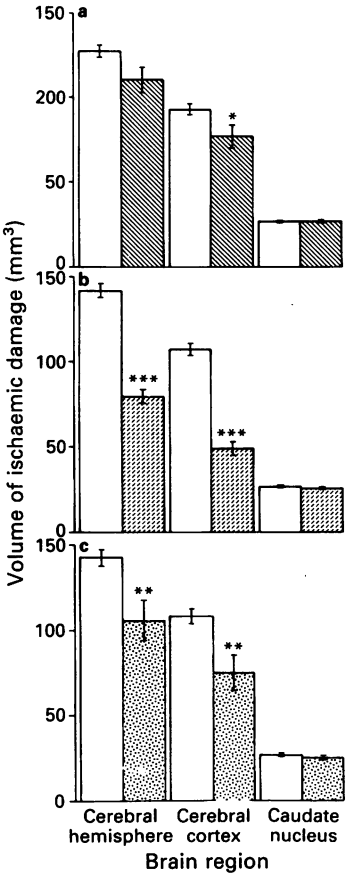

Figure 2 Volume of ischaemic damage in the cerebral hemisphere, cerebral cortex and caudate nucleus for the different doses of MK-801 and saline-treated animals (open columns). (a) Data for the 8.0 ng ml^{-1} plasma level of MK-801 (hatched columns). There was only significant protection against cortical damage, ANOVA $F = 4.5$ (* $P < 0.05$). There was no significant difference in the volume of ischaemic damage for the hemisphere (ANOVA $F = 4.13$, $P > 0.06$) or the caudate nucleus (ANOVA $F = 0.06$, $P > 0.8$). (b) Histogram for dose of MK-801 which gave plasma levels of 18.9 ng ml^{-1} (hatched columns). This gave significant protection against cortical (ANOVA $F = 111.63$, *** $P < 0.0001$) and hemispheric (ANOVA $F = 115.58$, *** $P < 0.0001$) ischaemic damage. However no protection against caudate damage was seen (ANOVA $F = 0.82$, $P > 0.4$). (c) The highest dose of MK-801 tested gave a plasma level of 113.2 ng ml^{-1} (stippled columns). This gave significant protection against cortical (ANOVA $F = 8.26$, ** $P > 0.01$) and hemispheric (ANOVA $F = 8.78$, ** $P < 0.01$) ischaemic damage. There was no protection against caudate (ANOVA $F = 1.19$, $P > 0.3$). Each column is mean for 9–10 animals, vertical bars show s.e.mean.

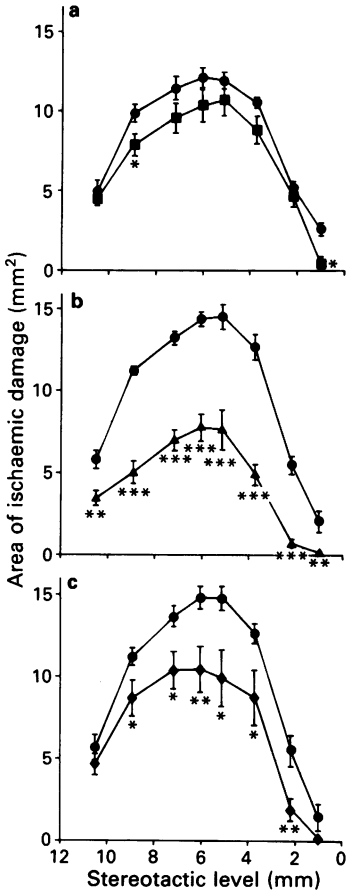

Figure 3 The area of ischaemic damage in the cerebral cortex at different stereotactic levels for the 3 doses of MK-801. (a) This illustrates data from the MK-801 group which had a plasma level of 8.0 ng ml^{-1} (■). The area of damage for the treated group was less than that for the saline-treated group (●) but there was no overall ($P > 0.05$) significant effect. Although statistical significance (* $P < 0.05$) was seen at 2 stereotactic levels. (b) The area of ischaemic damage for the 18.9 ng ml^{-1} dose of MK-801 (▲) was significantly ($P < 0.0001$) less than that for the saline-treated controls (●). There was a significant (*** $P < 0.0001$, ** $P < 0.01$) reduction in the ischaemic area at each stereotactic level. (c) The highest plasma concentration of MK-801 (113.2 ng ml^{-1}) (◆) also produced a signficant ($P < 0.01$) decrease in the overall area of ischaemic damage in the cerebral cortex. There was a significant (* $P < 0.05$, ** $P < 0.01$) decrease in the area of ischaemic damage for the MK-801-treated animals compared to the saline-treated controls (●) at coronal points between anterior 7.19 mm and anterior 2.18 mm from bregma. Each point represents the mean for 9–10 animals; vertical bars show s.e.mean.

MK-801 produced a marked hypotension in the rats. Immediately after administration of MK-801, MABP was reduced significantly ($P < 0.001$) to 60% of the preinjection level (MABP reduced from 99 ± 6 to 60 ± 2 mmHg; Figure 4c).

Discussion

The aim of the present experiments was to determine the minimum effective plasma level of MK-801 which was neuroprotective and to examine the dose-effect relationship of MK-801 in a focal ischaemia model. The acute model was used for these studies because it enabled critical physiological variables to be assessed repeatedly, such as MABP, $Paco_2$, Pao_2, and importantly the temperature of the animals which was maintained at 37 ± 0.5°C throughout the study. Furthermore, repeated blood samples were available enabling close monitoring of MK-801 plasma levels over the 4 h duration of the experiment. The infusion regimes were chosen to give a constant plasma level of MK-801 over the 4 h period, based on the assumption that the plasma half life of MK-801 in the rat is 1 h (Hucker *et al.*, 1982). The results of these studies indicate that the lowest effective plasma concentration of MK-801 is 8.0 ng ml^{-1} in this model because it produced a just significant reduction in the volume of cortical ischaemic cell damage. Maximum reduction (60%) in infarct size was seen with the plasma concentration of 18.9 ng ml^{-1}, this degree of infarct reduction correlates well with that observed by other investigators (Park *et al.*, 1988a,b; Oyzurt *et al.*, 1988). The plasma concentration of MK-801 of 113.2 ng ml^{-1} produced a 40% decrease in the infarct volume, which was less than that seen with a plasma concentration of 18.9 ng ml^{-1}. The reason for this may be due to the hypotensive effect of this dose of MK-801 as it has previously been shown that hypotension can increase infarct size in this model (Osborne *et al.*, 1987). Thus, the higher dose of MK-801 may result in a reduced protective effect as a result of its hypotensive action. However, the hypotensive effect of MK-801 is seen only in anaesthetized animals. In conscious, lightly restrained animals, MK-801 is hypertensive at these doses (Hargreaves, unpublished data) and this has also been reported in conscious rats following i.v. bolus doses (Lewis *et al.*,

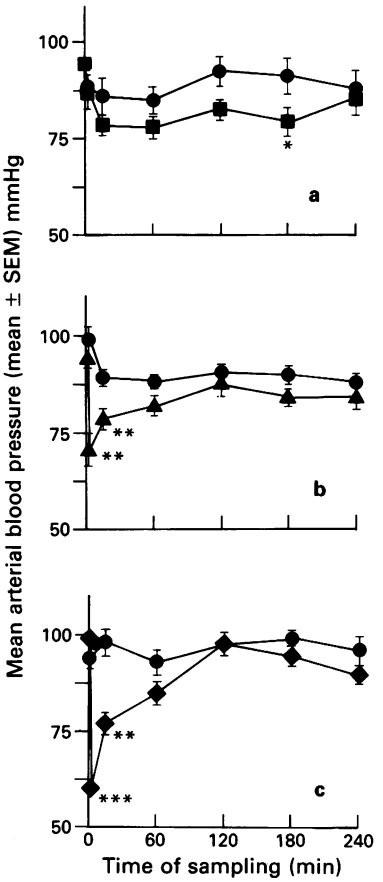

Figure 4 Changes in mean arterial blood pressure (MABP) produced by the 3 doses of MK-801 compared to their control groups. (a) Blood pressure effects produced by 8.0 ng ml^{-1} of MK-801 (■). There was no overall significant difference between the MK-801 and control group ($P = 0.2$) (●), although at the 2 h time point the MABP of the MK-801 group was significantly ($P < 0.05$) less than that of the control group. (b) MABP changes produced by a plasma concentration of 18.9 ng ml^{-1} of MK-801 (▲). The MABP was reduced significantly ($P = 0.006$) by 28 mmHg from the pre-injection level although it returned to level of controls (●) within 1 h. There was a significant (** $P < 0.01$) difference at 2 min and 15 min post MCA occlusion. (c) The highest dose of MK-801 (113.2 ng ml^{-1}) (◆) produced a marked hypotension in the rats. Following administration of MK-801, MABP was reduced significantly ($P = 0.004$) to 60% of the pre-injection level (MABP reduced from 99 to 60 mmHg). There was significant difference at 2 min (*** $P < 0.0001$) and 15 min (** $P < 0.001$) after MCA occlusion although the blood pressure had returned to same level as control animals (●) by 1 h. Values are mean with s.e.mean shown by vertical bars.

1989). There was no protection seen against caudate nucleus damage with any of the doses of MK-801, the reason for this being that the lenticulostriate branches are occluded in this model and these are the main blood supply to the caudate nucleus. Thus, with a much reduced blood flow no protection is seen in the caudate nucleus although the cell death is still through excitotoxic mechanisms.

The plasma levels of MK-801 required to protect against ischaemia-induced neuronal degeneration in the present study are the same as those required to block NMDA receptor mediated neurotoxicity *in vivo* (Willis *et al.*, 1991). The minimum effective plasma level of MK-801 of 8.0 ng ml^{-1} equals a concentration of 36 nM. This corresponds to the estimated K_D of MK-801 for the NMDA ion-channel site (Wong *et al.*, 1986; Huettner & Bean, 1988) and the concentration required to produce significant protection of cortical neurones in culture from hypoxia-induced degeneration (Priestley *et al.*, 1990). Given that MK-801 equilibrates well between plasma and cerebrospinal fluid (Willis *et al.*, 1991) with this dosing regime this provides further evidence to support the hypothesis that MK-801 mediates its neuroprotective action *in vivo* as a result of NMDA receptor blockade.

In the present experiments we have attempted to simulate some of the conditions that may prevail in the clinical situation. Thus, the drug was given as an i.v. bolus dose followed by a slow infusion to maintain stable plasma concentrations for the duration of the study. This was done using an acute model of stroke and therefore it could be argued that MK-801 is merely delaying ischaemic cell death. However, a recent study by Park (1988c) has shown that following permanent MCA occlusion and treatment with MK-801 the outcome is the same at 3 h or 48 h of survival. This is in agreement with the results of Nedergaard (1988), who found no delayed neuronal degeneration following permanent MCA occlusion in the rat. This is an important difference between global ischaemia models and permanent MCA occlusion in the rat.

MK-801 is a non-competitive NMDA antagonist which antagonizes the activated state of the NMDA receptor (Kemp *et al.*, 1987) by an open-channel blocking action. Other NMDA antagonists that act at the same site as MK-801, such as phencyclidine (Bielenburg, 1989) and thienylcyclohexylpiperidine (Duverger *et al.*, 1987), have also been shown to be neuroprotective in rat permanent MCA occlusion models. Furthermore a recent report has shown that (E)-4-(3-phosphonoprop-2-enyl)piperazine-2-carboxylic acid (CPPene), a competitive NMDA antagonist acting at the transmitter recognition site on the NMDA receptor complex, is neuroprotective in cats when administered prior to permanent MCA occlusion (Bullock *et al.*, 1990). Kynurenic acid which is a broad spectrum excitatory amino acid receptor antagonist acting at NMDA and non-NMDA subtypes of receptors (Evans *et al.*, 1987) was also neuroprotective in a rat MCA occlusion model (Germano *et al.*, 1987) when administered prior to the occlusion but was without effect on post-ischaemic administration. Ifenprodil, which has been reported to act at the polyamine site on the NMDA receptor complex (Carter *et al.*, 1989), is also neuroprotective in focal ischaemia models in the cat and rat (Gotti *et al.*, 1988). Recently, additional evidence for the involvement of excitatory amino acid induced neuronal damage in the rat MCA model has come from *in vivo* dialysis studies which have reported a substantial increase in extracellular glutamate and aspartate levels both in the striatum and cortex (Hillered *et al.*, 1989; Butcher *et al.*, 1990).

A recent report (Buchan *et al.*, 1989), however, has suggested that the neuroprotective action of MK-801 in focal ischaemia is due not to NMDA antagonism but to an increase in cerebral blood flow produced by the drug. The effect of MK-801 on cerebral blood flow in the same rat model of focal ischaemia as used in the present study was investigated by Park *et al.* (1989). They reported a significant decrease in cerebral blood flow produced by MK-801 in the non-ischaemic hemisphere but no effect was seen in the ischaemic hemisphere. Therefore, it is unlikely that the effects of MK-801 on cerebral blood flow contribute to its neuroprotective effects in this model.

MK-801 appears to be one of the most powerful neuroprotective agents available at the present time because it is able to ameliorate ischaemic damage when administered after the ischaemic insult in rats, cats and gerbils (Park *et al.*, 1988a,b; Ozyurt *et al.*, 1988; Gill *et al.*, 1988). Phencyclidine has also been shown to be neuroprotective when administered up to 3 h after permanent MCA occlusion (Bielenburg, 1989). Thus, it appears that non-competitive NMDA antagonists acting at the level of the ion channel linked to the NMDA receptor complex are effective on post-ischaemic administration. This is more relevant to the clinical situation giving a time window of up to 3 h for therapeutic intervention.

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R-(+)-HA-966, a glycine/NMDA receptor antagonist, selectively blocks the activation of the mesolimbic dopamine system by amphetamine

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1 The effects of the glycine/NMDA receptor antagonist, (+)-HA-966 on the neurochemical and behavioural responses to amphetamine have been determined in the mouse and rat.

2 In vehicle-treated control mice, (+)-HA-966 (30–100 mg kg^{-1}) did not affect dopamine synthesis in either the nucleus accumbens or striatum and was without marked effect on spontaneous locomotor activity.

3 In the mouse, (+)-HA-966 (30 and 100 mg kg^{-1}) dose-dependently blocked the enhancement of dopamine synthesis induced in the nucleus accumbens by amphetamine, but was without effect on the increase in dopamine synthesis in the striatum.

4 Intracerebroventricular administration of the glycine/NMDA receptor antagonist, 5,7-dichlorokynurenic acid, in the mouse (10 μg) also significantly attenuated amphetamine-enhanced DOPA accumulation in the nucleus accumbens, but not in the striatum.

5 The decrease of dopamine synthesis in striatum and nucleus accumbens induced by the dopamine receptor agonist, apomorphine, was unaffected by (+)-HA-966 (100 mg kg^{-1}).

6 (+)-HA-966 (30 mg kg^{-1}) failed to attenuate the hyperactivity induced by the systemic administration of amphetamine in the mouse, but totally prevented the hyperlocomotion following infusion of amphetamine into the rat nucleus accumbens. In contrast, stereotyped behaviour induced by infusion of amphetamine into the rat striatum was not altered following pretreatment with (+)-HA-966 (30 mg kg^{-1}).

7 The results are consistent with a selective facilitatory role of glycine/NMDA receptors on mesolimbic dopaminergic neurones.

Keywords: Glycine/NMDA receptor antagonists; (+)-HA-966; 5,7-dichlorokynurenic acid; dopamine synthesis; nucleus accumbens; striatum; locomotor activity; stereotypy

Introduction

Autoradiographic studies have revealed the presence of N-methyl-D-aspartate (NMDA) receptors in many forebrain structures, including the striatum and nucleus accumbens (Monaghan & Cotman, 1985; Maragos *et al.*, 1988) where dopaminergic neurones from the substantia nigra and ventral tegmentum terminate respectively. NMDA and other excitatory amino acid receptor agonists have been shown to stimulate the efflux of dopamine from striatum and nucleus accumbens, both *in vitro* and *in vivo* (Roberts & Sharif, 1978; Roberts & Anderson, 1979; Jhamandas & Marien, 1987; Carter *et al.*, 1988; Kalivas *et al.*, 1989; Shimuzu *et al.*, 1990), while the increase in rat locomotor activity induced by infusion of NMDA into the nucleus accumbens can be attenuated by depletion of brain dopamine with reserpine, or by administration of dopamine receptor antagonists (Donzanti & Uretsky, 1983). Thus, the NMDA receptor appears to play a facilitatory role in the regulation of nigrostriatal and mesolimbic dopaminergic systems.

The NMDA receptor is now recognised to comprise multiple modulatory sites giving rise to at least four distinct classes of NMDA receptor antagonists, viz: those acting (i) competitively at the neurotransmitter recognition site; (ii) by blocking the associated ion channel; (iii) by antagonizing the potentiating actions of polyamines; and (iv) by antagonizing the potentiating actions of glycine (for review see Lodge & Johnson, 1990). R-(+)-HA-966 (R-(+)-3-amino-1-hydroxypyrrolid-2-one) is an antagonist at the glycine/NMDA modulatory site (Singh *et al.*, 1990a). The profile of this compound is distinct from NMDA ion channel blockers, such as phencyclidine (PCP) (Anis *et al.*, 1983), in that it does not generalise to PCP in drug discrimination paradigms, stimulate motor activity or alter dopamine metabolism (Singh *et al.*, 1990a,b; Tricklebank & Saywell, 1990). In the present paper it is shown that (+)-HA-966 is able to attenuate the neurochemical and behavioural response to the dopamine releasing agent, amphetamine, in both the rat and mouse. Furthermore, this effect of (+)-HA-966, whilst prominent in the nucleus accumbens, is absent from the striatum.

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Methods

Animals

Male Sprague-Dawley rats (250–350 g) and male BKTO mice (20–30 g) (Bantin and Kingman, Hull) were housed in groups of five or six respectively on a 12 h light:dark cycle (lights on 06 h 00 min) and allowed standard laboratory diet and tap water *ad libitum*.

Neurochemical studies

Catecholamine synthesis in striatum and nucleus accumbens was determined by measurement of the accumulation of 3,4-dihydroxyphenylalanine (DOPA) following inhibition of aromatic amino acid decarboxylase with 3-hydroxybenzylhydrazine (NSD 1015, Carlsson *et al.*, 1972). Mice were injected with either saline (0.9% NaCl, 10 ml kg^{-1}, i.p.) or (+)-HA-966 (30 and 100 mg kg^{-1}, i.e. 258 and 860 μmol kg^{-1}, i.p.), followed 20 min later with either saline (10 ml kg^{-1}, s.c.) or (+)-amphetamine sulphate (2.5 mg kg^{-1}, s.c.). Thirty min later all mice were injected with NSD 1015, (100 mg kg^{-1}, i.p.) and

killed by stunning and decapitation after a further 30 min. In a separate experiment mice were pretreated with either saline (10 ml kg^{-1}, i.p.) or (+)-HA-966 (100 mg kg^{-1}, i.p.) followed 10 min later with either saline (10 ml kg^{-1}, s.c.) or apomorphine HCl (1 mg kg^{-1}, s.c.). Twenty min later all mice were injected with NSD 1015 (100 mg kg^{-1}) and killed after a further 30 min.

In order to determine the effect of 5,7-dichlorokynurenic acid, a glycine receptor antagonist which does not readily penetrate the blood brain barrier, on amphetamine-enhanced catecholamine synthesis, mice were anaesthetized with halothane and the antagonist injected (10 μg (38.4 nmol) in 5 μl of distilled water) intracerebroventricularly (i.c.v.) (Singh *et al.*, 1990a). Control mice were injected with 5 μl water. These animals were then administered (+)-amphetamine and NSD 1015 at the doses and times described above. Following decapitation, brains were removed and the striatum and nucleus accumbens dissected with a steel punch. Samples were stored at −70°C. Tissue was then homogenized in 10 vol 0.4 M perchloric acid containing 0.1% sodium metabisulphate, 0.01% EDTA, and 0.1% cysteine and centrifuged at 3000 *g* for 20 min. The supernatant was subjected to high performance liquid chromatography (h.p.l.c.) with electrochemical detection. The system comprised an h.p.l.c. Technology Techsphere 3 μm ODS column (4.6 mm × 7.5 cm). The mobile phase consisted of 0.07 M KH_2PO_4, 0.0035% EDTA, 0.023% octyl sodium sulphate and 12.5% methanol, pH 2.75. This was filtered (0.2 μm) and degassed with helium before use. DOPA content was determined with an ESA Coulochem model 5100A electrochemical detector (Severn Analytical Ltd) with a dual electrode analytical cell (model 5011). Electrode 1 was set at −0.04 V and electrode 2 at +0.42 V. Under these conditions separation of DOPA (retention time, 2.3 min), DOPAC (3.8), dopamine (4.4), 5-hydroxyindoleacetic acid (5-HIAA) (6.5), 5-hydroxytryptophan (5-HTP) (8.1), homovanillic acid (HVA) (9.0), and 5-hydroxytryptamine (5-HT) (11.8) was achieved.

Stereotaxic surgery

Rats were anaesthetized with sodium pentobarbitone (Sagatal, RMB; 60 mg kg^{-1}, i.p.) and implanted bilaterally with guide cannulae (23 gauge) positioned either 0.5 mm above the nucleus accumbens (AP + 1.7 mm; L ± 1.5 mm from bregma; V − 5.5 mm from dura) or 1 mm above the striatum (AP + 0.2 mm; L ± 3.4 mm from bregma; V − 6 mm from dura; Paxinos & Watson, 1982). Stainless steel stylets (31 gauge) were used to keep the guide cannulae patent throughout the experimental procedure.

Intracerebral infusion

Two days after surgery the stylets were removed and infusion needles (31 gauge) inserted bilaterally into, and terminating 0.5 mm (accumbens) or 1 mm (striatum) below, the guide cannulae. Drug solutions or saline were infused at a rate of 0.5 μl per min into the nucleus accumbens or striatum respectively with a Carnegie Medicin CMA/100 infusion pump (Biotech Inst.). A total volume of 0.5 μl was infused into each accumbens or 1 μl into each striatum and the infusion needles left in place for 60 s before replacing the stylets. Rats received four infusions in a latin square design with a two day recovery period between testing.

Mouse locomotor activity

Mice were habituated for 2 h to individual activity cages (230 × 280 × 210 mm) lined with wire grids to permit climbing and equipped with two parallel infra red beams at the base of the cage to record activity (horizontal beam breaks) and cage crossing (consecutive beam breaks). Two more beams were positioned at the top of the cage to monitor climbing activity. The mice were pretreated with either saline (10 ml kg^{-1}) or (+)-HA-966 (30 mg kg^{-1}, s.c.) followed 30 min later by either saline (10 ml kg^{-1}, s.c.) or (+)-amphetamine (5 mg kg^{-1}, s.c.) and the number of photocell beams breaks occurring in 10 min intervals recorded for 2 h. In a separate experiment, the effects of (+)-HA-966 on spontaneous activity was determined by injecting non-habituated mice with either saline (10 ml kg^{-1}, s.c.) or (+)-HA-966 (10 or 30 mg kg^{-1}, s.c.) 30 min before being placed in the activity cages. Locomotor activity was recorded for 2 h.

Rat locomotor activity

Rats were habituated for 3 h to individual activity cages (300 × 200 × 300 mm) containing five infra-red beams: two at the top of the cage to record rearing activity and three at the base for recording total activity (horizontal beam breaks) and cage crossing (consecutive beam breaks). Animals were pretreated with either saline (1 ml kg^{-1}), (+)-HA-966 (30 mg kg^{-1}, i.p.) or haloperidol (0.003–0.3 mg kg^{-1}, s.c.). Thirty min later, rats were treated with either saline (1 ml kg^{-1}, s.c.) or (+)-amphetamine (0.75 mg kg^{-1}, s.c.) systemically or 10 μg in a total volume of 1 μl (0.5 μl min^{-1} per side) infused directly into the nucleus accumbens. Control animals received the same volume of saline. Activity was then recorded over 2 h as previously described. In a separate experiment, to determine the effect of (+)-HA-966 on spontaneous activity, non-habituated rats were treated with either saline (1 ml kg^{-1}, i.p.) or (+)-HA-966 (3–30 mg kg^{-1}, i.p.) and returned to the home cage. Thirty min later, the animals were placed in individual activity cages. Activity was recorded for 2 h.

Rat behavioural observation

Rats were habituated for 30 min to individual perspex observation cages (300 × 200 × 200 mm) and then pretreated with either saline (1 ml kg^{-1}), (+)-HA-966 (30 or 60 mg kg^{-1}, i.p.) or haloperidol (0.01–0.3 mg kg^{-1}, s.c.). Thirty minutes later all rats were treated with (+)-amphetamine (10 mg kg^{-1}, s.c.) and the duration of sniffing and oral stereotypy (licking/biting) assessed in two 5 min observation periods, 45–50 and 60–65 min following (+)-amphetamine injection.

In a separate experiment, the effects of haloperidol and (+)-HA-966 on stereotypy induced by intra-striatal infusion of amphetamine were investigated. Following a 30 min habituation period, rats were pretreated with either saline (1 ml kg^{-1}), haloperidol (0.03 or 0.1 mg kg^{-1}, s.c.) or (+)-HA-966 (30 mg kg^{-1}, i.p.) and bilaterally infused 30 min later with either saline (1 μl per side) or (+)-amphetamine (40 μg per side in a volume of 1 μl) into the striatum. The duration of sniffing and oral stereotypy (licking/biting) was then recorded for the following 30 min.

Histological verification

Following the final infusion, rats were killed, brains removed and infusion sites confirmed histologically. Rats with needle tracts terminating outside the nucleus accumbens or medial striatum were excluded from the study.

Statistical analysis

Results were analysed by analysis of variance and, where appropriate, between group comparisons were made by either Tukey's or Dunnett's test. In some cases, dose-response relationships were evaluated by trend analysis.

Drugs

Apomorphine HCl, (Sigma), 3-hydroxybenzylhydrazine (NSD 1015, Sigma), (+)-amphetamine sulphate (Sigma) and (+)-HA-966 (R-(+)-3-amino-1-hydroxypyrrolid-2-one, synthesized in the Department of Chemistry, MSD, Terlings Park) were

dissolved in 0.9% NaCl. 5,7-Dichlorokynurenic acid was also synthesized at Terlings Park and was dissolved in water. Haloperidol was used as the commercially available form, Haldol (Janssen).

Results

Effect of (+)-HA-966 on amphetamine-enhanced DOPA accumulation in mouse striatum and nucleus accumbens

(+)-Amphetamine (2.5 mg kg^{-1}, s.c.) significantly enhanced DOPA accumulation in striatum and nucleus accumbens by 67 and 37% respectively when compared with saline-treated mice. Pretreatment with (+)-HA-966 (30 and 100 mg kg^{-1}, i.p.) did not affect DOPA accumulation in either brain region of saline-treated mice, but dose-dependently decreased the enhancement of DOPA accumulation induced by amphetamine in the nucleus accumbens. In contrast, (+)-HA-966 did not alter the amphetamine-induced accumulation of DOPA in the striatum (Figure 1).

Effect of 5,7-dichlorokynurenic acid on amphetamine-enhanced DOPA accumulation in mouse striatum and nucleus accumbens

The increase of DOPA accumulation produced by (+)-amphetamine (2.5 mg kg^{-1}, s.c.) in striatum and nucleus accumbens was similar to the previous experiment. Pretreatment with 5,7-dichlorokynurenic acid (10 μg, i.c.v.) did not affect DOPA accumulation in either brain region of saline-treated mice, but significantly decreased the enhancement of DOPA accumulation by amphetamine in the nucleus accumbens. As with the experiment using (+)-HA-966, 5,7-dichlorokynurenic acid did not affect amphetamine stimulated DOPA accumulation in striatum (Figure 2).

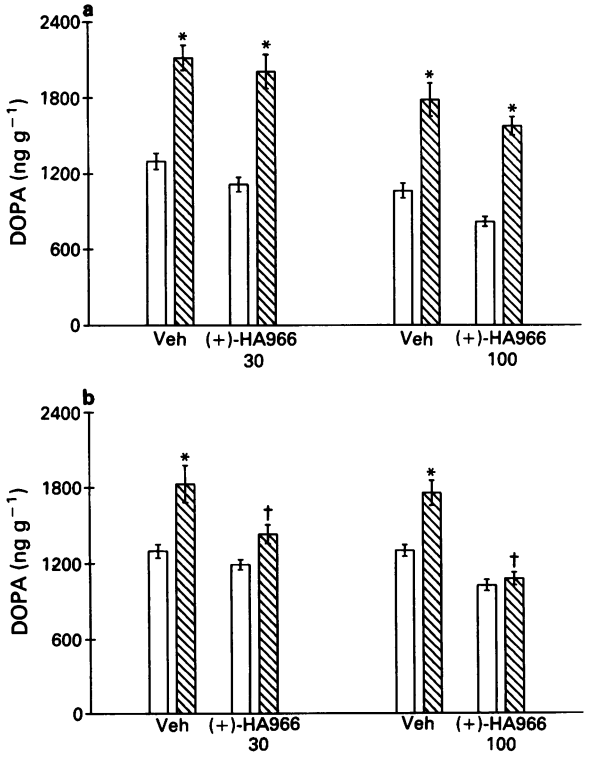

Figure 1 Effects of (+)-HA-966 at 30 and 100 mg kg^{-1} i.p. on amphetamine-stimulated DOPA accumulation in the mouse (a) striatum and (b) nucleus accumbens. Open columns show the effects of pretreatment with either saline or (+)-HA-966 in saline-treated mice and cross-hatched columns show the effects of pretreatment with either saline or (+)-HA-966 in mice treated with amphetamine. Each column shows the mean for 5–6 animals; vertical bars show s.e.mean. Data were analysed by two-way ANOVA followed by Tukey's test; * $P < 0.01$ compared with appropriate control group, † $P < 0.05$ compared with vehicle-treated animals given amphetamine.

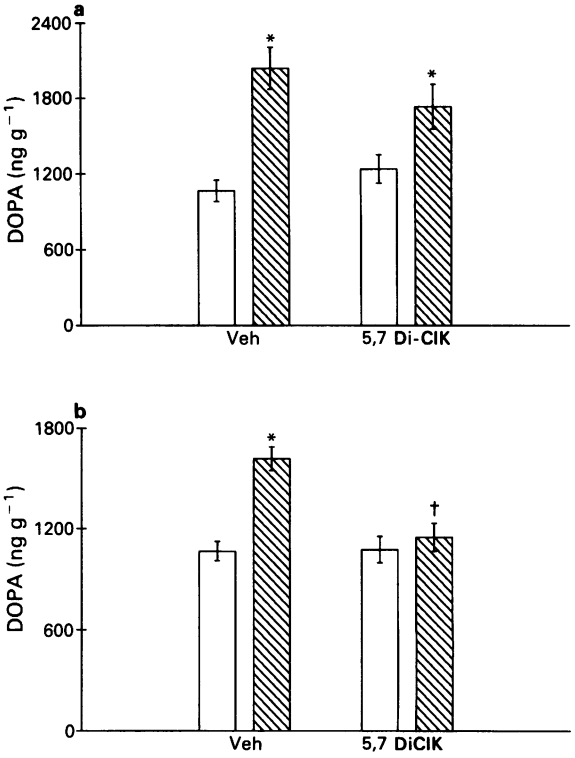

Figure 2 Effects of 5,7-dichlorokynurenic acid (5,7-DiClK, 10 μg per mouse, i.c.v.) on amphetamine-stimulated DOPA accumulation in mouse striatum (a) and nucleus accumbens (b). Open columns show the effect of pretreatment with either saline or 5,7-DiClK in saline-treated mice and cross-hatched columns show the effects of pretreatment with either saline or 5,7-DiClK in mice treated with amphetamine. Each column shows the mean for 5–6 animals; vertical bars show s.e.mean. Data were analysed by two-way ANOVA followed by Tukey's test, * $P < 0.01$ compared with appropriate control group; † $P < 0.05$ compared with vehicle-treated animals given amphetamine.

Effect of (+)-HA-966 on the apomorphine-induced decrease of DOPA accumulation in mouse striatum and nucleus accumbens

Apomorphine (1 mg kg^{-1}, s.c.) caused a pronounced and significant decrease of DOPA accumulation in both the striatum and nucleus accumbens. As before, (+)-HA-966 (100 mg kg^{-1}, i.p.) was without effect on striatal or nucleus accumbens DOPA accumulation in vehicle-pretreated mice. However, in contrast to its inhibitory effects on amphetamine-enhanced dopamine synthesis, (+)-HA-966 did not block the inhibitory effect of apomorphine in either brain region (Figure 3).

Effect of (+)-HA-966 on spontaneous activity in non-habituated mice and rats

The effect of (+)-HA-966 on spontaneous motor activity was assessed 30 min after injection in both mice (10 or 30 mg kg^{-1}, s.c.) and rats (3–30 mg kg^{-1}, i.p.). In mice, all activity measurements recorded (i.e. horizontal beam breaks, crossings and climbing) were similar to those representing normal exploratory activity in saline-pretreated animals (Figure 4a). In the rat, pretreatment with (+)-HA-966 (30 mg kg^{-1}, i.p.) significantly attenuated rearing behaviour, but was without effect on the number of cage crossings or horizontal beam breaks (Figure 4b).

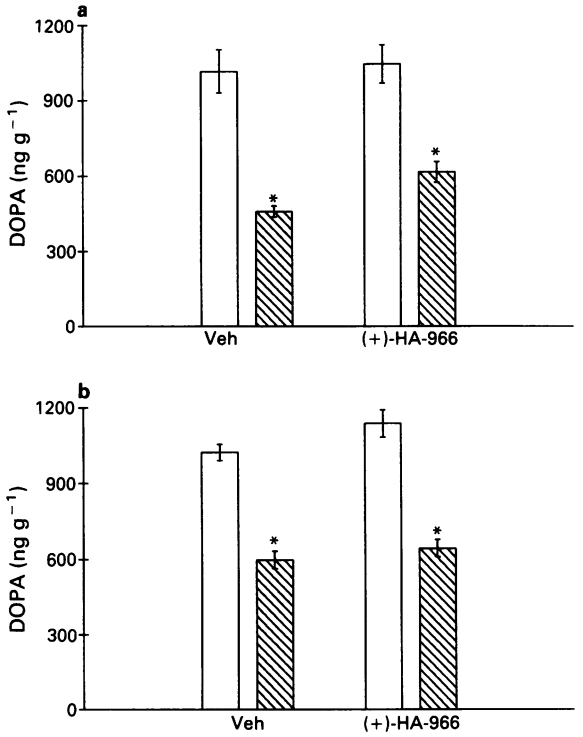

Figure 3 Effects of (+)-HA-966 at 100 mg kg^{-1} i.p. on apomorphine-induced decrease of DOPA accumulation in the mouse striatum (a) and nucleus accumbens (b). Open columns show the effect of pretreatment with either saline or (+)-HA-966 in saline-treated animals and cross-hatched columns show the effects of pretreatment with either saline or (+)-HA-966 in mice treated with apomorphine. Each column shows the mean of 5–6 animals; vertical bars show s.e.mean. Data were analysed by two-way ANOVA followed by Tukey's test. * $P < 0.01$ compared with appropriate control group.

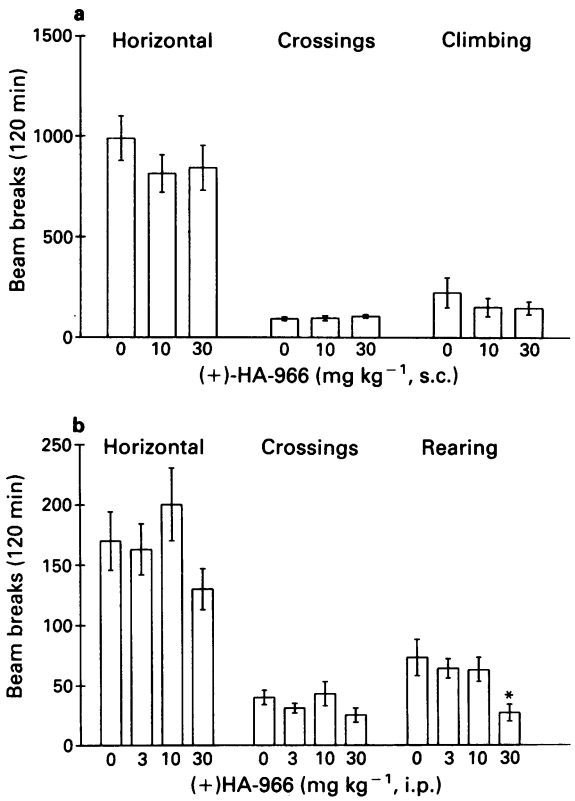

Figure 4 Effect of (+)-HA-966 on spontaneous motor activity recorded 30 min following injection in (a) mice (10 or 30 mg kg^{-1}, s.c.; $n = 8$ per group) and (b) rats (3–30 mg kg^{-1}, i.p.; $n = 10$ per group). Results are expressed as mean activity counts (s.e.mean shown by vertical bars) for horizontal beam breaks, cage crossings and top beam breaks (i.e. climbing in mice or rearing in rats) recorded over a 2 h observation period. Data for each behavioural parameter were analysed by one-way analysis of variance followed by Dunnett's t test comparing all groups to saline-treated animals; * $P < 0.05$.

Effect of (+)-HA-966 on hyperactivity induced by systemically administered (+)-amphetamine in the mouse

Preliminary studies showed that administration of (+)-amphetamine (2.5–10 mg kg^{-1}, s.c.) to mice previously habituated to individual activity cages significantly and dose-dependently increased horizontal beam breaks and the frequency of cage crossing compared to saline-pretreated animals (data not shown). The lowest dose of (+)-amphetamine to induce a robust and reliable increase in beam breaks and cage crossing was 5 mg kg^{-1}. (+)-HA-966 (30 mg kg^{-1}, s.c.) was without effect on the motor response of this dose of (+)-amphetamine (Figure 5).

Effect of (+)-HA-966 on hyperactivity induced by systemically administered (+)-amphetamine in the rat

As with previous studies in mice, administration of (+)-amphetamine (0.375–1.5 mg kg^{-1}, s.c.) to rats significantly and dose-dependently stimulated locomotor activity as reflected by an increase in horizontal beam breaks and cage crossing frequency (data not shown). An increase in rearing behaviour was also observed, although this failed to reach statistical significance when compared to the response in saline-treated animals (data not shown). Pretreatment with haloperidol (0.003–0.3 mg kg^{-1}, s.c.) dose-dependently antagonized the increase in horizontal beam breaks ($ED_{50} = 0.015$ mg kg^{-1}) and cage crossings ($ED_{50} = 0.017$ mg kg^{-1}) induced following a sub maximal dose of (+)-amphetamine (0.75 mg kg^{-1}, s.c.) data not shown. Pretreatment with (+)-HA-966 (30 mg kg^{-1}, i.p.) significantly attenuated, but did not abolish, the hyperactivity induced by amphetamine (0.75 mg kg^{-1} s.c.): the increase in total horizontal beam breaks and cage crossings recorded in 2 h was reduced by 44% and 33% respectively (Figure 6).

Effect of (+)-HA-966 on hyperactivity induced by infusion of (+)-amphetamine into the nucleus accumbens

Administration of saline (0.5 μl per side) into the nucleus accumbens of rats pretreated with either saline or (+)-HA-966 (30 mg kg^{-1}, i.p.) caused a small increase in activity during the first 20 min which declined over the remainder of the 2 h recording period (Figures 7 and 8). In contrast, infusion of (+)-amphetamine (5–20 μg per rat) bilaterally into the rat nucleus accumbens caused a pronounced and dose-dependent increase in horizontal beam breaks, cage crossings and rearing which was maximal 10–20 min after infusion and declined to control values by 60 min (Figures 7 and 8). However, when a submaximal dose of (+)-amphetamine (10 μg per rat) was infused into the nucleus accumbens of animals pretreated with (+)-HA-966 (30 mg kg^{-1}, i.p.), values for horizontal beam breaks, cage crossing and rearing were not significantly different from those of animals given only saline (Figure 8).

Effect of (+)-HA-966 on stereotyped behaviour induced by systemic administration of (+)-amphetamine in the rat

Subcutaneous injection of a high dose of (+)-amphetamine (10 mg kg^{-1}) in the rat induced a pronounced stereotyped behavioural response consisting of continuous sniffing and licking/biting behaviours. Haloperidol (0.01–0.3 mg kg^{-1}, s.c.)

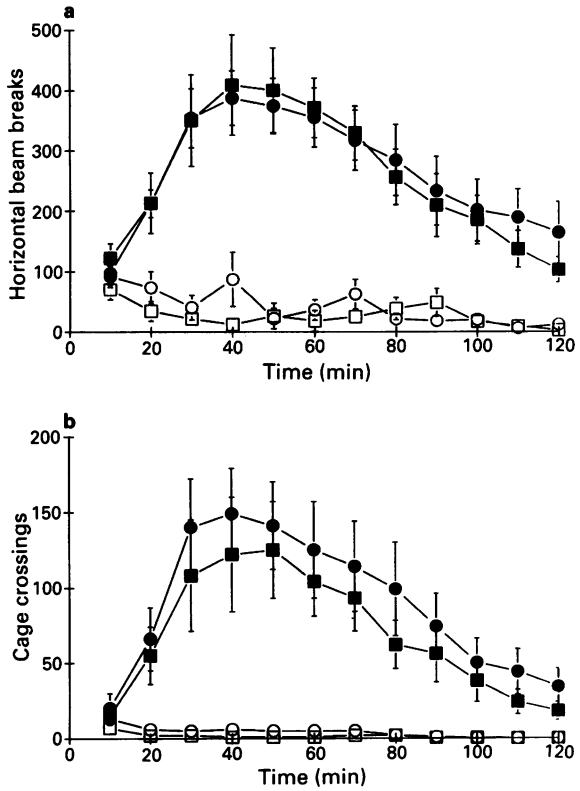

Figure 5 Effect of 30 min pretreatment with (+)-HA-966 on amphetamine-induced increase in (a) horizontal beam breaks and (b) cage crossings in mice; (○) saline (10 ml kg^{-1}, s.c.) + saline (10 ml kg^{-1}, s.c.); (●) saline (10 ml kg^{-1}, s.c.) + (+)-amphetamine sulphate (5 mg kg^{-1}, s.c.); (□) (+)-HA-966 (30 mg kg^{-1}, s.c.) + saline (10 ml mg^{-1}, s.c.); (■) (+)-HA-966 (30 mg kg^{-1}, s.c.) + (+)-amphetamine sulphate (5 mg kg^{-1}, s.c.). Results are expressed as mean activity counts (s.e.mean shown by vertical bars, $n = 8$ per group). Data were analysed by analysis of variance for repeated measures and showed no significant effect of (+)-HA-966 on amphetamine-induced hyperactivity responses.

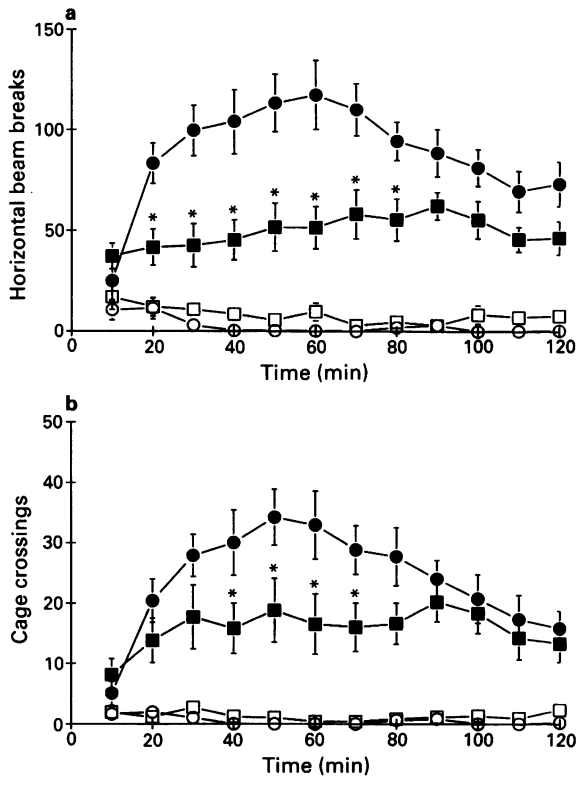

Figure 6 Effect of 30 min pretreatment with (+)-HA-966 on (+)-amphetamine sulphate-induced increase in (a) horizontal beam breaks and (b) cage crossings in rat; (○) saline (1 ml kg^{-1}, i.p.) + saline (1 ml kg^{-1}, s.c.); (●) saline (1 ml kg^{-1} i.p.) + (+)-amphetamine sulphate (0.75 mg kg^{-1}, s.c.); (□) (+)-HA-966 (30 mg kg^{-1}, i.p.) + saline (1 ml kg^{-1}, s.c.); (■) (+)-HA-966 (30 mg kg^{-1}, i.p.) + (+)-amphetamine sulphate (0.75 mg kg^{-1}, s.c.). Results are expressed as mean activity counts (s.e.mean shown by vertical bars, $n = 10$ per group). Data were analysed by analysis of variance for repeated measures followed by Tukey's *post hoc* test; * $P < 0.05$ compared to saline + (+)-amphetamine treated rats.

given 30 min before amphetamine, dose-dependently antagonized both amphetamine-induced sniffing ($ED_{50} = 0.12$ mg kg^{-1}) and licking/biting responses ($ED_{50} = 0.01$ mg kg^{-1}, Figure 9a). In contrast, pretreatment with (+)-HA-966 (30 and 60 mg kg^{-1}, i.p.) failed to attenuate (+)-amphetamine induced licking and biting behaviour (Figure 9b) and the decrease in sniffing seen with (+)-HA-966 (60 mg kg^{-1}) was not statistically significant even after analysis of the data by trend analysis ($P > 0.05$). Although variability in this experiment was greater than in Figure 9a and some attenuation of the behaviour with (+)-HA-966 cannot be excluded, the high dose required is considerably in excess of that required to abolish totally hyperactivity induced by the intra-accumbens infusion of amphetamine.

Effect of (+)-HA-966 on stereotyped behaviour induced by infusion of (+)-amphetamine bilaterally into rat striatum

Bilateral infusion of (+)-amphetamine (40 μg per side in a volume of 1 μl) into the rat striatum induced a robust and significant increase in the duration of sniffing (mean ± s.e.mean, 966 ± 115 s) and licking/biting (431 ± 110 s) compared to behavioural responses observed following saline infusions (177 ± 32 and 4 ± 2 s respectively). Furthermore, pretreatment with haloperidol (0.03 and 0.1 mg kg^{-1}, s.c.) dose-dependently and significantly attenuated amphetamine-induced stereotypy at doses similar to those effective in blocking both the hyperlocomotion and stereotypy observed following systemic (+)-amphetamine injection (Figure 10a). In contrast, pretreatment with (+)-HA-966 (30 mg kg^{-1}, i.p.) did not significantly attenuate either (+)-amphetamine-induced sniffing or oral stereotypies induced following infusion into the rat striatum (Figure 10b).

Discussion

Results in the present study provide neurochemical and behavioural evidence that (+)-HA-966 selectively interacts

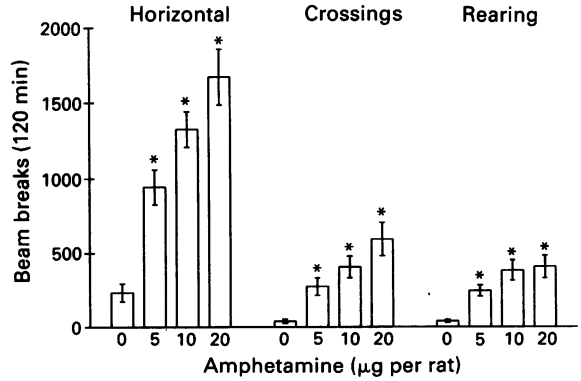

Figure 7 Effect of bilateral infusion of (+)-amphetamine sulphate (5–20 μg per rat) into the rat nucleus accumbens on total horizontal beam breaks, cage crossings and rearing recorded for 120 min following drug administration. Results are expressed as mean activity counts (s.e.mean shown by vertical bars, $n = 12$) and all rats received all treatments in a latin square design. Data were analysed by analysis of variance for repeated measures followed by Dunnett's t test comparing all groups to activity responses following saline infusion; * $P < 0.05$.

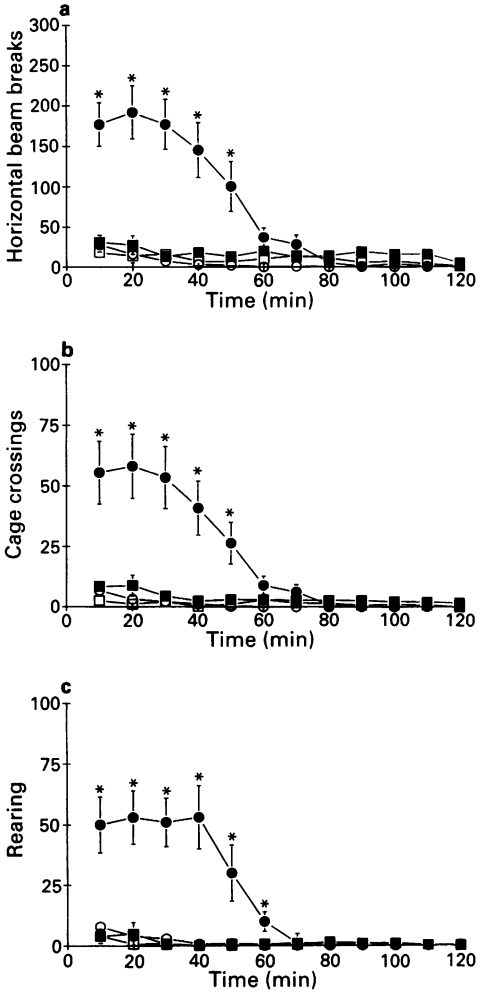

Figure 8 Effect of 30 min pretreatment with (+)-HA-966 on the increase in (a) horizontal beam breaks, (b) cage crossings and (c) rearing induced by bilateral infusion of (+)-amphetamine sulphate into rat nucleus accumbens; (○) saline (1 ml kg^{-1}, i.p.) + saline (0.5 μl per side); (●) saline (1 ml kg^{-1}, i.p.) + (+)-amphetamine sulphate (5 μg in 0.5 μl per side); (□) (+)-HA-966 (30 mg kg^{-1}, i.p.) + saline (0.5 μl per side); (■) (+)-HA-966 (30 mg kg^{-1}, i.p.) + (+)-amphetamine sulphate (5 μg in 0.5 μl per side). Results are expressed as mean activity counts (s.e.mean shown by vertical bars, $n = 9$) and each rat received all treatments in a latin square design. Data were analysed by analysis of variance for repeated measures followed by Tukey's *post hoc* test; * $P < 0.05$ compared to saline/saline-treated rats.

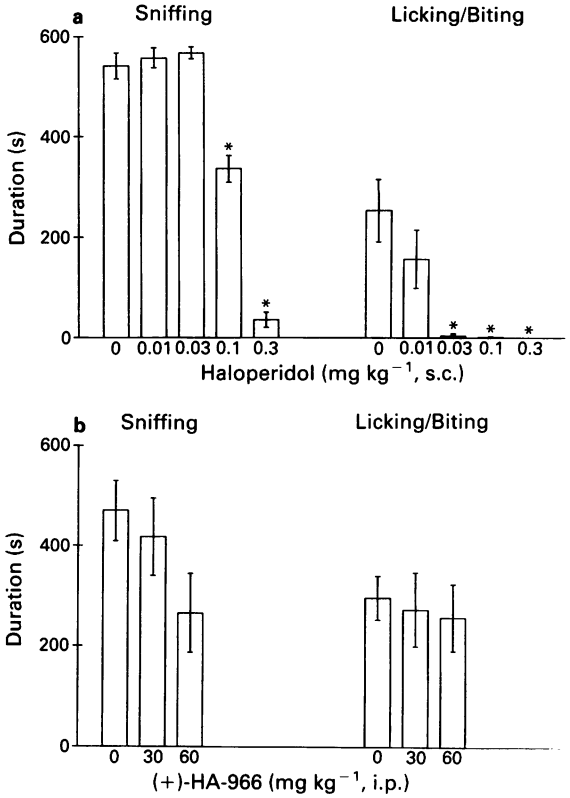

Figure 9 Effect of 30 min pretreatment with (a) haloperidol (0.01–0.3 mg kg^{-1}, s.c., $n = 8$ per group) and (b) (+)-HA-966 (30 or 60 mg kg^{-1}, i.p., $n = 8$–10 per group) on (+)-amphetamine (10 mg kg^{-1}, s.c.) induced stereotypy in rats. Results are expressed as the mean (s.e.mean shown by vertical bars) duration of sniffing and oral stereotypy (licking/biting) assessed in two 5 min observation periods, 45–50 and 60–65 min following (+)-amphetamine injection. Data were analysed by one-way analysis of variance followed by Dunnett's t test comparing all groups to vehicle pretreated rats, * $P < 0.05$. The effect of (+)-HA-966 on amphetamine-induced sniffing was also analysed by linear trend analysis.

with mesolimbic, but not nigrostriatal dopaminergic neurones. Thus, in the absence of any direct effects on brain dopamine synthesis, as estimated by the accumulation of DOPA following aromatic amino acid decarboxylase inhibition (Carlsson *et al.*, 1972), (+)-HA-966 selectively and dose-dependently blocked amphetamine-stimulated dopamine synthesis in the nucleus accumbens, but not in the striatum. The lack of effect of (+)-HA-966 on dopamine synthesis *per se* is in accord with our previous finding that although (±) and (−)-HA-966 increased striatal dopamine concentration in a manner similar to that caused by γ-butyrolactone, (+)-HA-966 was without effect (Singh *et al.*, 1990a).

(+)-HA-966 selectively inhibits responses to the activation of NMDA receptors by an antagonist action at the glycine modulatory site on the NMDA receptor/ion channel complex. In contrast, the (−)-enantiomer of HA-966 only weakly interacts with the glycine/NMDA receptor and is more potent in causing sedation than the (+)-enantiomer (Singh *et al.*, 1990a). Unlike the non-competitive NMDA receptor antagonists such as phencyclidine (PCP) or MK-801, (+)-HA-966 did not itself alter mesolimbic dopamine turnover (present study), induce PCP-like stereotypy (Tricklebank & Saywell, 1990) or generalize to the PCP discriminative stimulus (Singh *et al.*, 1990b). Indeed preliminary evidence indicates that (+)-HA-966 can antagonize both the increase in mesolimbic dopamine turnover and the motor stimulation induced by PCP (Hutson *et al.*, 1990; Bristow *et al.*, 1990). While (+)-HA-966 blocks the hyperactivity induced by systemically administered PCP (which activates cortico-limbic, but not striatal dopamine metabolism, Hutson *et al.*, 1990), it did not block the hyperactivity induced by systemically administered amphetamine in the mouse and produced only a modest attenuation in the rat. In view of the regionally selective neurochemical effects of (+)-HA-966, one explanation for this lack of behavioural antagonism may be that the response to systemically administered amphetamine (as monitored by photocell activity) is influenced by both limbic and non-limbic dopamine pathways which are comparably activated by amphetamine.

Consequently, the ability of (+)-HA-966 to block the motor response to amphetamine infused directly into either the nucleus accumbens or the striatum was examined. Following intra-accumbens amphetamine infusion, (+)-HA-966 totally abolished the resultant hyperactivity response. In contrast, stereotypy induced by intra-striatal or systemically administered amphetamine was not significantly affected by (+)-HA-966. It could be argued that the lack of effect of (+)-HA-966 on stereotypy induced by intrastriatal infusion of amphetamine could reflect the (necessary) use of higher doses of amphetamine than those required to induce hyperactivity following injection into the nucleus accumbens. However, since haloperidol was, if anything, more potent in blocking the behavioural response to striatal infusion of amphetamine than

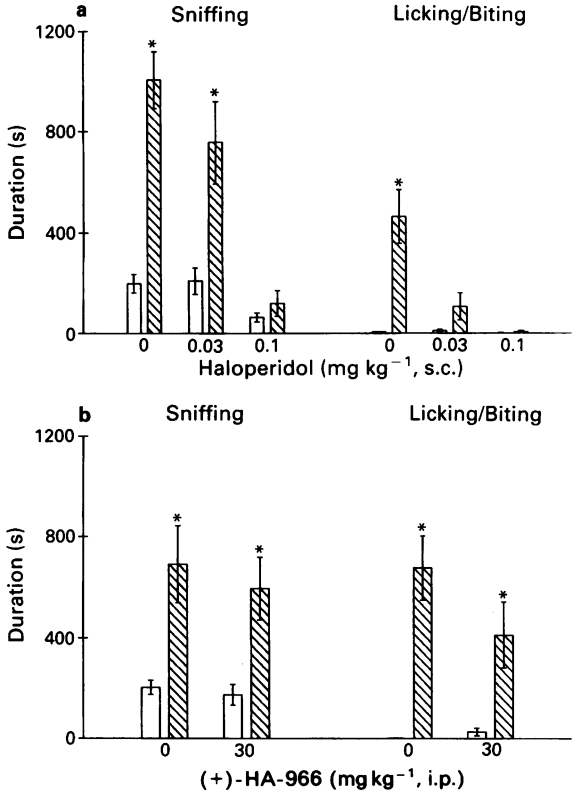

Figure 10 Effect of 30 min pretreatment with (a) haloperidol (0.03 or 0.1 mg kg^{-1}, s.c., $n = 6$ or 7) or (b) (+)-HA-966 (30 mg kg^{-1}, i.p., $n = 10$) on the stereotyped behavioural response induced following bilateral infusion of (+)-amphetamine sulphate (40 μg μl^{-1} per side) into rat striatum. Results are expressed as the mean (s.e.mean shown by vertical bars) duration of sniffing and oral stereotypy (licking/biting) determined from 0–30 min following saline infusion (open columns) or (+)-amphetamine infusion (hatched columns). Data were analysed by analysis of variance for repeated measures followed by Dunnett's t test comparing (+)-amphetamine responses to those seen following saline infusion; * $P < 0.05$.

in antagonizing the response to the systemic injection of the stimulant, this seems unlikely. These findings thus support the neurochemical studies demonstrating that (+)-HA-966 selectively attenuates mesolimbic, but not striatal dopamine responses.

The inhibitory effect of (+)-HA-966 on amphetamine-enhanced dopamine synthesis in the nucleus accumbens and behavioural hyperactivity is consistent with the previously described facilitatory role for glutamate on dopaminergic neurones (Roberts & Anderson, 1979; Donzanti & Uretsky, 1983; Carter *et al.*, 1989; Kalivas *et al.*, 1989). However it is notable that the majority of previous neurochemical studies have been concerned with dopamine/glutamate interactions in the striatum. This region, apart from receiving a major dopamine input from the substantia nigra is also innervated by glutamate containing neurones from the frontal cortex. Thus, lesions of the cortex decrease striatal glutamate concentration (Kim *et al.*, 1977; Fonnum *et al.*, 1981) and reduce the number of high affinity glutamate uptake sites in striatal synaptosomes (Divac *et al.*, 1977; McGeer *et al.*, 1977). It is surprising, therefore, to see no effect of (+)-HA-966 on striatally-mediated neurochemical and behavioural responses to amphetamine.

The nucleus accumbens is also innervated by glutamate-containing neurones arising in the hippocampus and amygdala (Kelley & Domesick, 1982; Kelley *et al.*, 1982; Christie *et al.*, 1987). Several studies have shown that intra-accumbens infusion of excitatory amino acid receptor agonists such as NMDA, glutamate, quisqualate, and kainate (Donzanti & Uretsky, 1983; O'Neill *et al.*, 1987) induces hyperlocomotion which is presumably mediated by increased dopamine release since it is blocked by reserpine and dopamine receptor antagonists (Donzanti & Uretsky, 1983). Furthermore, the hyperactivity induced by the systemic administration of amphetamine or cocaine was attenuated by infusion of the AMPA-selective receptor antagonist, glutamyl diethyl ester (GDEE), directly into the nucleus accumbens (Pulvirenti *et al.*, 1989).

As (+)-HA-966 blocked the effects of the dopamine releaser, amphetamine but not the directly acting dopamine receptor agonist, apomorphine, on dopamine synthesis it is likely that (+)-HA-966 interacts at pre-junctional sites on the dopamine neurone. Binding sites for the NMDA/glycine receptor complex are present in striatum and nucleus accumbens to a similar extent (Monaghan & Cotman, 1985; Maragos *et al.*, 1988; McDonald *et al.*, 1990). However, the apparent regionally selective interaction of (+)-HA-966 with dopaminergic neurones could reflect the differential distribution of NMDA/glycine receptors on dopaminergic neurones in these brain regions. Thus, extensive 6-hydroxydopamine (6-OHDA) lesions of the nigro striatal system did not significantly affect the number of high affinity [^{3}H]-PCP binding sites in the striatum (Snell *et al.*, 1984), whereas quinolinic or kainic acid lesions of the striatum reduced binding to NMDA receptors by 92% (Greenamyre & Young, 1989; Samuel *et al.*, 1990) suggesting that their location is predominantly postsynaptic with respect to dopaminergic neurones in the striatum. In contrast, extensive 6-OHDA lesions of the nucleus accumbens or the ventral tegmental area (VTA) depleted nucleus accumbens dopamine concentration and high affinity [^{3}H]-PCP binding sites by approximately 70% suggesting that in the nucleus accumbens, a large proportion of NMDA receptors are located presynaptically on dopamine terminals (French *et al.*, 1985). However, it is conceivable that [^{3}H]-PCP is labelling dopamine reuptake sites and/or sigma recognition sites in these regions (Largent *et al.*, 1986; Vignon *et al.*, 1988) and therefore, studies using the more selective radioligand, [^{3}H]-MK-801, are in progress to determine the differential distribution of the NMDA receptor complex in these regions.

Modulation of mesolimbic dopamine metabolism by (+)-HA-966 could also occur at dopamine cell bodies within the VTA. Dopaminergic neurones project from this nucleus predominantly, though not exclusively, to the mesolimbic system including the nucleus accumbens. Excitatory amino acid receptors within the VTA have been demonstrated electrophysiologically *in vivo* (Greenhoff *et al.*, 1988) and *in vitro* (Seutin *et al.*, 1990). In addition, Kalivas *et al.* (1989) has shown that infusion of glutamate into the VTA enhances dopamine release in the nucleus accumbens and increases locomotor activity.

In conclusion, the results demonstrate that (+)-HA-966 in the absence of effects on dopamine neurotransmission *per se*, can modulate the activation by amphetamine of mesolimbic, but not striatal dopamine systems. Whether the interaction of (+)-HA-966 with mesolimbic dopaminergic neurones is mechanistically based on the glycine receptor or some other as yet undefined pharmacological action is not yet known. However, the similarly selective action of 5,7-dichlorokynurenic acid, a structurally unrelated NMDA/glycine receptor antagonist, on amphetamine-enhanced mesolimbic dopamine synthesis is consistent with an involvement of the glycine receptor.

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Corrigendum

Br. J. Pharmacol. (1990), **101**, 908–912

L.A. Chahl. Effects of putative neurotransmitters and related drugs on withdrawal contractures of guinea-pig isolated ileum following brief contact with [Met^5] enkephalin

In this paper, the drug cyclo(7-amino heptanoyl-Phe-D-Trp-Lys-Thr [Bzl]) was not in fact of the cyclised form and therefore not a somatostatin antagonist. The conclusions derived from the results obtained with this drug are not valid.
In all other respects, the data and conclusions are still correct.

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